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JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY
Volume 39, Supplement 1, March 1952

DISCUSSION ON RADIATION MICROBIOLOGY AND BIOCHEMISTRY

PUBLISHED BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
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1952

FOREWORD

Concerning Supplements to the

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A large part of the cost of most scientific journals is borne by the subscribers. The publication of lengthy manuscripts in the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY would require a further increase in the subscription price or, alternatively, would reduce the number of pages which could be assigned to other authors who desire to publish articles relating to the many fields of physiology encompassed by this Journal. Accordingly, in the interest of our subscribers, it is necessary to limit the number of pages made available to any author and for the presentation of material dealing with any one subject.

Despite these considerations, certain authors rightly desire to publish the results of their research in an extensive form at a cost which cannot fairly be assessed against the subscribers; certain observations and data may require such a form of publication. To satisfy these desires and needs the Editorial Board of the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY has approved, on a trial basis, the publication of occasional Supplements to the Journal. The articles published in these Supplements must meet the usual standards of scientific merit; the authors must provide the full cost of publication.

It is hoped that such a Supplement will fulfill the requirements for unique publications and will supply scientific reports of unusual interest to our subscribers at no additional cost to them.

DETLEV W. BRONK

DISCUSSION ON RADIATION MICROBIOLOGY AND BIOCHEMISTRY

GIVEN AT

RESEARCH CONFERENCE FOR BIOLOGY AND MEDICINE OF THE ATOMIC ENERGY COMMISSION

sponsored by

THE BIOLOGY DIVISION
OAK RIDGE NATIONAL LABORATORY

Oak Ridge, Tennessee
April 11, 12, 1949

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CONTENTS

ABSTRACTS OF PAPERS

INTRODUCTION

The second research conference of the Division of Biology and Medicine of the Atomic Energy Commission and the Biology Division of the Oak Ridge National Laboratory was held in Oak Ridge on April 11 and 12, 1949.

This conference dealt with the effects of radiation at the microbiological level. As in the previous year, free discussion was encouraged. The meeting was very successful, with approximately 250 scientists attending. The following speakers took part: James H. Jensen, Howard B. Newcombe, C. P. Swanson, Albert Kelner, S. E. Luria, Renato Dulbecco, Walter M. Dale, R. E. Zirkle, G. E. Stapleton, and L. H. Gray.

Each of the speakers except one submitted a manuscript. Unfortunately, there was considerable delay in obtaining some of these papers. The authors had an opportunity to review their manuscripts before they went to press.

A COMPARISON OF SPONTANEOUS AND INDUCED MUTATIONS OF ESCHERICHIA COLI TO STREPTOMYCIN RESISTANCE AND DEPENDENCE

HOWARD B. NEWCOMBE

National Research Council of Canada, Atomic Energy Project, Chalk River, Ontario

TWO FIGURES

Much of the interest in the production of gene changes by irradiation has arisen from the striking similarity between induced mutants and those which occur spontaneously. However only very limited information is available concerning the extent of the similarity.

That there are differences in the proportions of gross chromosomal rearrangements, minute deletions, and true gene mutations arising from spontaneous, ultraviolet-induced, and γ-induced change suggests that gene mutations alone might be shown to vary with the circumstances of their origin if suitable materials were available for such a study.

For a critical comparison of spontaneous and induced mutations one might use either a single quantitative character, or a group of qualitatively different characters. In either case appreciable numbers of spontaneous and induced mutations would be studied, and the proportions of the various detectably different phenotypes would be determined. That such a comparison has not already been carried out using the classical genetic materials is presumably due to the difficulty of obtaining a sufficient number of spontaneous mutants of independent origin affecting a suitable character or group of characters.

In the present experiments it has been possible to follow this approach by using a readily detectable group of changes

in the bacterium *Escherichia coli,* namely, those to streptomycin resistance and dependence. These characters are suited to the purpose since they include both qaulitative and quantitative variants. The mutation pattern and the experiments comparing spontaneous, ultraviolet-induced, and γ-induced change will be described.

STREPTOMYCIN RESISTANCE AND DEPENDENCE

E. coli B/r mutates in a single step to a high level of streptomycin resistance, and the mutant forms are readily selected from large populations of sensitive cells by plating in the presence of the drug. The concentration is not critical since as little as 32 units (1 μg = 1 unit) per cm^3 will inhibit the parent strain and any partially resistant variants, while the fully resistant forms can grow in concentrations as high as 50,000 units per cm^3.

The change from sensitivity to full resistance takes place spontaneously at the relatively low rate of 2×10^{-10} per bacterium per division cycle, as measured by the standard methods of Luria and Delbrück ('43), (Scott, '49; Newcombe and Hawirko, '49). Similar changes are induced in appreciable numbers by irradiation with ultraviolet or with γ rays.

The mutant forms have been divided into three main categories: simple resistant (sr), partially dependent (sd_o), and fully dependent (sd_x). Strains of the first category grow normally both in the absence and in the presence of streptomycin; those of the second give rise to very minute colonies in the absence of the drug but grow normally in the presence of low concentrations (about 2 to 4 units per cm^3); those of the third will not form visible colonies unless streptomycin is present in appreciable concentrations, the usual minimum being in the region of 32 to 128 units per cm^3.

The fully dependent (sd_x) form is capable of mutating to a number of different phenotypes, including: sensitivity (that is, the reverse phenotypic change), partial dependence, and simple resistance, as indicated in figure 1. In addition, forms possessing intermediate degrees of resistance have been

obtained from the sd_x strains but have not yet been studied in detail. These changes occur both spontaneously and as the result of irradiation, and can be selected by plating suspensions of mutants in the absence of streptomycin or with very low concentrations of the drug.

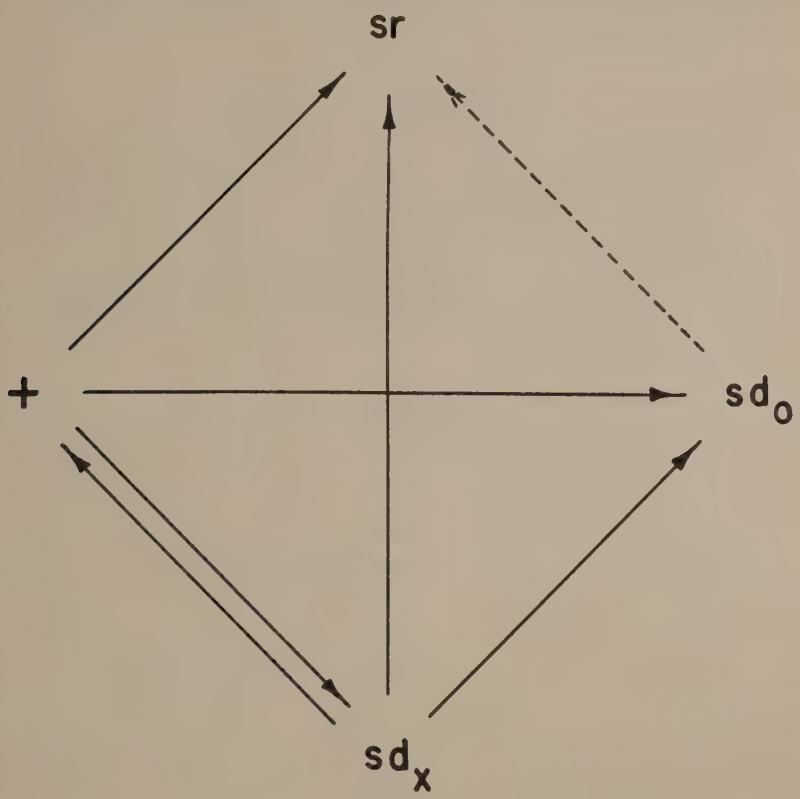

Figure 1

The partially dependent form also mutates to independence. Nothing is known, however, of changes occurring in the resistant form, and no means have yet been found for selecting sensitive or dependent variants from resistant populations.

It will be seen that mutations of this group lend themselves to the present study in a number of ways. First, there is a variety of forms and the proportions in which these occur

as the result of spontaneous and of induced mutation may be compared. Second, the properties of the sd_x mutants can be expressed numerically in terms of the concentration of streptomycin required for normal growth. Third, there is positive means of selecting both the forward mutations from + to sd_x, and the reverse changes. The present study deals only with the forward changes.

METHODS

Irradiation-induced mutations to streptomycin resistance, like those to phage resistance, cause no immediate phenotypic change in the bacteria in which they occur, except possibly in rare instances. Thus if one is to determine the number of induced mutations in a treated suspension it is necessary that the bacteria be permitted a period of growth before applying the selective agent.

Samples of a treated suspension were plated with 2 cm^3 of melted soft agar (0.7%) on the surface of nutrient agar plates. When this had hardened, an additional 2 cm^3 of soft agar was poured on top of it to prevent any rearrangement of the bacteria during subsequent treatment. The plates were incubated at 37°C. for periods ranging from 0 to 7 hours in 1-hour steps, and at the end of this preliminary incubation, streptomycin was applied in the form of a third 2-cm^3 layer of soft agar containing 10,000 units of the drug. To prevent any further growth from taking place until the streptomycin had diffused through to the bacteria, the plates were chilled and stored overnight at 5°C. They were then incubated 96 hours and colony counts made.

This technique is similar in principle to that developed by Demerec ('46) for the detection of induced mutations to phage resistance. In both cases the descendants of a single irradiated bacterium are confined to one locality on the plate, and each colony arising after treatment with the selective agent represents a separate induced mutation.

It was found that a preliminary incubation period of approximately 4 hours was required for all the induced mutations

to be phenotypically expressed; and, where plates were subject to longer periods than this, no further increase in colony count was observed. With excessively long preliminary incubation (7 hours or more) the streptomycin-sensitive cells became so numerous as to interfere with the subsequent development of the resistant colonies and a decline in colony count was observed. Subsequent experiments have shown that varying the period of chilling within the range from 2 to 40 hours had no effect on the plate counts or on the proportions of colonies belonging to the three categories of resistance.

In all experiments considerably numbers of the colonies were tested to determine the proportions of mutations to the sr, sd_o, and sd_x forms. Colonies were grown in broth plus streptomycin (500 units per cm^3), and a 1:100 dilution of these suspensions streaked on plain and on streptomycin agar (containing 1000 units per cm^3). Scorings were made after two days of incubation.

A G. E. Germicidal lamp was used for the irradiation with ultraviolet light (this delivers 95% of its ultraviolet energy in the 2537 Å line), dose being measured with a Westinghouse dose meter. Gamma radiation was from a radioactive cobalt (Co^{60}) source delivering approximately 9000 r per hour to the material as measured chemically. Spontaneous mutations were obtained using a similar technique starting with untreated bacterial suspensions.

Samples from a single large culture grown in synthetic medium (M9 of Anderson, '46) and stored at 5°C., were used throughout. The spontaneous mutant colonies were from two separate experiments carried out prior to and after the radiation experiments; the three categories of resistance occurred in similar proportions in both cases.

EXPERIMENTAL RESULTS

Proportion of changes to the related phenotyes, sr, sd_o, and sd_x. The relative frequencies of mutations to sr, sd_o, and sd_x are shown in tables 1, 2, and 3, for spontaneous, ultraviolet-induced, and γ-induced change respectively. Only a slight

difference was observed between spontaneous and γ-induced mutation in this respect, the relative frequencies being close to 2:2:1 in both cases. Ultraviolet-induced mutation on the

TABLE 1

Spontaneous mutations to the three categories of streptomycin resistance

BACTERIA [a] PLATED	RESISTANT COLONIES (Totals from 100 plates each)	RATIO ($sr:sd_o:sd_x$)	PERCENTAGES ($sr:sd_o:sd_x$)
5×10^4	33	8:20:5	24:61:15
5×10^5	94	37:45:12	39:48:13
5×10^6	242	91:114:37	38:47:15
Total	369	136:179:54	37:48:15

[a] All plates were incubated 5 hours before the application of streptomycin.

TABLE 2

Ultraviolet-induced mutations to the three categories of streptomycin resistance

(Dose = 500 ergs per mm², survival = 40%)

INCUBATION	RESISTANT COLONIES (per 10^8 plated) [a]	RATIO (from 20 colonies tested) ($sr:sd_o:sd_x$)
(hours)		
0	0	.....
1	25	4:5:11
2	134	6:4:10
3	302	5:4:11
4	857	5:2:13
5	837	2:6:12
6	770	5:4:11
7	... [b]	.....
Total		27:25:68
Percentage		22:21:57

[a] 3×10^7 and 3×10^8 bacteria were plated for each incubation period; the above values are calculated from the plates having between 50 and 1000 colonies.

[b] Few resistant colonies visible against the dense background of sensitive cells.

other hand was associated with a much higher proportion of sd_x's, the ratio being approximately 1:1:2. This difference is consistent throughout all observations.

The extent to which the precise conditions of the experiment might alter the ratio has been studied by varying (a) the

preliminary incubation period between irradiation and selection (tables 2 and 3), (b) the degree of crowding at the time of selection (tables 1, 2, and 3), (c) the concentration of streptomycin used for selection (table 4), and (d) the concentration of streptomycin in which the resistant colonies were grown prior to testing (data not shown).

Selection with low concentrations of streptomycin increased the proportion of sr's, presumably by favoring sr variants

TABLE 3

Gamma-induced mutations to the three categories of streptomycin resistance

(Survival for the two doses = 36 and 7% respectively)

	9000 r		18,000 r	
INCUBATION	RESISTANT COLONIES (per 10^8 plated)[a]	RATIO (from 20 colonies) ($sr:sd_o:sd_x$)	RESISTANT COLONIES (per 10^8 plated)[b]	RATIO (from 20 colonies) ($sr:sd_o:sd_x$)
0	0.5	.....	3	.....
1	2	.....	4	.....
2	8	4:10:6	14	4:11:5
3	16	8:7:5	28	9:8:3
4	18	6:8:6	51	6:10:4
5	14	9:8:3	51	6:7:7
6	13[c]	8:9:3	48	8:7:5
7	4[c]	12:8:0	17[c]	13:2:5
Total		47:50:23		46:45:29
Percentage		39:42:19		38:38:24

[a] 2×10^8 viable bacteria plated; average of two plates.

[b] 5×10^7 viable bacteria plated; average of two plates.

[c] Few resistant colonies visible against the dense background of susceptible cells.

within the developing sd_x colonies. In a range of high concentrations, however, there were no detectable differences. The other variables tested seemed to have no influence on the proportions, although from subsequent experiments it would appear that crowding sometimes favors sr's. This effect is small and apparently insufficient to account for the observed differences although, until more is known of the metabolic requirements of the mutant forms, these diffierences must be interpreted with caution.

A comparison of spontaneous and induced variants within the sd_x category. Spontaneous and induced sd_x's were compared quantitatively with regard to the concentration of streptomycin required for normal colony formation under standard conditions. To do this dilutions of the cultures from the original mutant colonies (containing approximately 1000 bacteria) were plated with melted agar to which streptomycin had been added in concentrations ranging in twofold steps from one to 1024 units per cm^3. These plates were incubated

TABLE 4

Effect of streptomycin concentration upon number of colonies and ratio of the three categories of resistant mutants

(Ultraviolet-treated material; dose = 500 ergs per mm^2)

STREPTOMYCIN CONCENTRATION (relative)[a]	RESISTANT COLONIES (per 10^8 plated)[b]	RATIO (from 60 colonies each) ($sr:sd_0:sd_x$)
0.17	674	22:26:12
0.25	706	8:27:25
0.50	656	6:18:36
1.00	758	6:14:40
Total		42:85:113

[a] Expressed as a fraction of the concentration used in other experiments. Relative concentrations lower than 0.17 gave very large numbers of colonies.

[b] 3×10^7 viable bacteria plated.

4 days, and the minimum concentration necessary for the bulk of the cells to form colonies of countable size was recorded.

The results are shown in figure 2. In all cases an appreciable proportion of the mutant strains required between 32 and 128 units per cm^3. There were, however, few which required only two or 4 units, and these will be seen to be most numerous in the γ-induced sd_x's, and least numerous in the ultraviolet-induced sd_x's. Ultraviolet thus seems to be associated with low proportions of sr's and, within the two categories of dependents, with the presence of few mutants which could grow in concentrations of less than 8 units per cm^3. Here again it is not entirely certain to what extent differences in

the degree of crowding may have influenced the proportions of the various types. It should be noted, however, that crowding was greatest in the case of the spontaneous mutations while the proportion of sd_x's with a low streptomycin requirement was greatest in the γ-induced mutants. This would suggest that the effect is not an artifact.

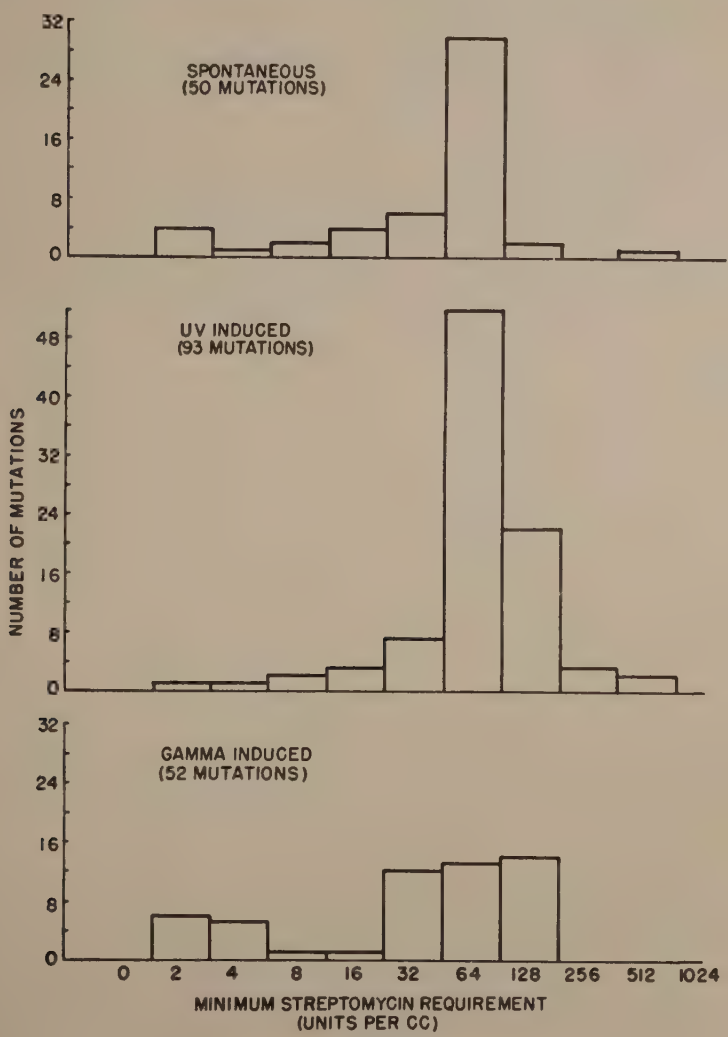

Figure 2

Spontaneous and induced mutability with respect to two unrelated changes. Prior to the present experiments only one mutation (or group of mutations) of *E. coli*, namely, to phage T1 resistance, had been studied with respect both to the spontaneous mutation rate and to the yields of induced mutants per unit dose of ultraviolet and γ or X rays. In table 5 this information has been compared with the corresponding values for streptomycin resistance. For the comparison, the proportions of mutations to streptomycin and to phage resistance

TABLE 5

Mutability to streptomycin resistance and to phage resistance. A comparison of spontaneous, ultraviolet-induced and gamma- or X-ray-induced mutation

MUTATION TO	SPONTANEOUS (per bact. per div. cycle) $\times 10^{-8}$	ULTRAVIOLET (per bact. per erg per mm^2) $\times 10^{-8}$	GAMMA OR X RAY (per bact. per r unit) $\times 10^{-8}$
Streptomycin resistance	0.02 [a]	2 [b]	0.005 [b]
Phage T1 resistance	3 [c]	20 [d]	0.2 [d]
Ratio	1:150	1:10	1:40

[a] From Newcombe and Hawirko, '49.
[b] From tables 2 and 3.
[c] From Newcombe, '48.
[d] From Demerec and Latarjet, '46.

have been determined for changes arising in the three ways. Spontaneous and ultraviolet-induced changes would appear to be widely different and γ-induced change intermediate.

DISCUSSION

In the foregoing, spontaneous and induced mutation have been compared with regard to the proportions of various distinguishable phenotypes. For the sake of convenience these phenotypes have been divided into three groups:

(a) related (sr, sd_o, and sd_x),

(b) closely related (the quantitative variants within the sd_x category),
(c) unrelated (phage resistance, and streptomycin resistance),

each of which has been considered separately. In all groups, the proportions of the various types of mutants associated with one or the other of the two mutagenic agents have differed appreciably from the proportions characteristic of spontaneous change. This could conceivably be an artifact due to selection, but attempts to demonstrate selective influences which would account for the observed differences have so far failed.

Assuming that the differences are genuine, it is of interest that they are relatively small. Considerably greater dissimilarities in the genetic effects of various mutagenic agents have been encountered when major chromosome rearrangements were taken into consideration. X (and presumably γ) irradiation, for example, produces a much higher ratio of gross chromosomal changes to minute rearrangements and gene mutations than does ultraviolet (Stadler, '41). The chemical mutagens (mustards, diepoxides, and peroxides) produce chromosome breaks which are quite differently distributed along the chromosomes of *Vicia faba* than those caused by X rays (C. E. Ford, '49; Revell, unpublished quoted by Ross, '50; for other differences see Auerbach, '50). Short ultraviolet produces the so-called "K mutations" (changes of unknown genetic origin) of *Chaetomium globosum* while X rays and long ultraviolet do not (McAulay and J. M. Ford, '47; J. M. Ford and Kirwan, '49). On the basis of these observations it might have been expected that the present comparisons would have yielded differences of similar magnitude. Instead, however, a quite striking similarity in the end products of spontaneous, ultraviolet-induced, and γ-induced gene mutation has been indicated.

At the time that these experiments were carried out it was not certain how many gene loci were involved in the various changes of streptomycin response. Recent crossing experi-

ments using Lederberg's ('47) lines of *E. coli* strain K-12 have shown that there is a single locus which mutates to different allelic forms controlling resistance and dependence (Demerec, '50; Newcombe and Nyholm, '50). If we accept the differences indicated by the present comparisons, this would mean not only that the various loci respond differently to different mutagenic influences, but that the direction of mutation within a single locus to its various allelic forms may be influenced by the agent responsible for the change. Mutagenic agents would thus appear to show both inter- and intragenic specificities.

In all the experiments of this nature there is the difficulty of distinguishing gene mutations from gene losses (see Stadler and Roman, '48). However, in the present, presumably haploid, organism it seems unlikely that individuals which had suffered genetic loss would have survived to form viable mutant lines. In addition it is known that the dependent forms can undergo further mutational changes in streptomycin response, and that in the majority of cases these involve a second mutation at the original locus (Newcombe and Nyholm, '50). This would imply that we are dealing with gene mutation rather than gene loss, although the possibility of successive partial losses is not entirely ruled out.

SUMMARY

Spontaneous, ultraviolet-induced, and γ-induced mutations have been compared in *E. coli* using the changes from streptomycin sensitivity to resistance and dependence. These mutants were divided into three categories, sr, sd_o, and sd_x; and the sd_x category was further subdivided into a range of types requiring different concentrations of the drug.

Mutants arising from ultraviolet irradiation contained a higher proportion of the sd_x category than did those from spontaneous or γ-induced change. Minor differences were noted between mutations occurring spontaneously and those arising from γ irradiation. From the control experiments it appears unlikely that these differences are due to selection

although, until more is known of the metabolic requirements of the various mutant forms, critical evidence will be lacking.

It would appear, from comparison of the mutation rates to streptomycin and to phage resistance, that different loci are differentially sensitive to different mutagenic agents.

Since the streptomycin variants are known to occur mainly through mutation at a single locus, it is suggested that there may be mutagenic specificities which are both inter- and intragenic.

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THE EFFECT OF SUPPLEMENTARY FACTORS ON THE RADIATION-INDUCED FREQUENCY OF MUTATIONS IN ASPERGILLUS TERREUS[1]

C. P. SWANSON
The Johns Hopkins University, Baltimore, Maryland

ONE FIGURE

INTRODUCTION

One of the important but unsolved problems of cytogenetics is the relationship of gene mutations to chromosomal changes. Analysis of the giant chromosomes of *Drosophila* has indicated that chromosomal rearrangements having phenotypic expressions which are inherited in Mendelian fashion may grade down in size until they approach submicroscopic dimensions, thus making difficult any accurate description of mutation in terms of intra- or extragenic alteration of structure. Solution of the problem is complicated by the fact that the usual mutagenic agents cause both chromosomal changes and mutations. A possible avenue of approach, however, is through the use of supplementary agents which, while ineffective alone, can, when combined with X rays or short ultraviolet, increase or decrease the frequency of chromosomal changes without affecting the induction of mutations, or vice versa. The data herein reported summarize the results obtained through such experiments.

A strain of *Aspergillus terreus* has been used for all the experiments. Its ease of handling, its constancy of colonial form and color, and its regular mutability when exposed to radiation or chemical mutagens, combine to make it a particularly desirable organism for the preliminary study of certain as-

[1] Supported by a grant-in-aid from the National Institutes of Health, U. S. Public Health Service.

pects of mutation induction. Its lack of a known sexual stage, however, imposes limitations on its usefulness, limitations which can be overcome, however, by the use of such fungi as *Neurospora* for genetic analysis.

The procedures followed in radiation experiments have been previously described in detail (Swanson, Hollaender, and Kaufmann, '48), and require no further elaboration except where departures have been made to suit particular experiments. These departures are explained in their appropriate place.

INFRARED AS A SUPPLEMENTARY TREATMENT FOR MUTATION INDUCTION

The data on the frequencies of morphological mutations obtained when spores of *Aspergillus*, which had been previously exposed to infrared radiation, were then irradiated by X rays or ultraviolet (2537Å) have been published (Swanson, Hollaender, Kaufmann, '48). For purposes of comparison with data from other combination treatments, the results are briefly summarized. Infrared, by itself, was without mutagenic effect, some 740 isolations having no morphological mutations of a detectable nature. The strain of *Aspergillus* used in these experiments was likewise quite stable, no spontaneous mutations arising in the several years that the strain has been under observation. When the infrared, applied as a pretreatment, was combined with X rays, a significantly higher rate of mutation was obtained throughout the range of dosages employed (8500–170,000 r). Of the various types of morphological mutations obtained, none in particular seemed to be increased by the infrared, but rather a general rise in frequency of all types was obtained.

When combined with ultraviolet, the infrared appears to be without effect on the frequency of mutations at the lower dosages of ultraviolet (see fig. 2, Swanson, Hollaender, and Kaufmann, '48). The rate of recoverable mutations at the peak of the curve was definitely greater, however, with the decline in

frequency at higher dosages following, in a parallel fashion but at a somewhat higher level, that of the ultraviolet controls.

Without attempting to make a distinction between those phenotypic changes which result from point mutations and those associated with chromosomal breakage, these data seemed best interpreted by assuming that the infrared affected the mutation process when combined with X rays, but not when combined with ultraviolet. The rise in frequency at the peak of the ultraviolet curve, and beyond, was assumed to result from the absorption of infrared by the spores which led to a greater differential survival rate of mutant types. This might be a nongenic effect which would counteract and/or repair the deleterious cytoplasmic effect of the ultraviolet in a manner similar to that proposed, on the basis of incubation studies, by Hollaender and Emmons ('41), and which would lead to a selective survival of mutants at the higher doses of ultraviolet.

NITROGEN MUSTARD AS A SUPPLEMENTARY TREATMENT FOR MUTATION INDUCTION

It is now well established that the nitrogen mustards are mutagenic on a wide variety of phylogenetically diverse organisms. In *Aspergillus*, spores from a 10-day-old culture suspended for 30 minutes in a 1.0% aqueous (unbuffered) solution of mustard (bis-β-chloroethylmethylamine-HCl) yielded 1.5% of morphological mutations. A 0.1% concentration was without appreciable mutagenic effect when handled in the same manner (Swanson and Goodgal, '48). That this lack of effect of the lower concentration was not a matter of penetration, but one simply of ineffectiveness when used alone, was made evident by data obtained from a series of mustard-ultraviolet combination treatments. When spores pretreated for 30 minutes with a 0.1% concentration of mustard were subsequently exposed to ultraviolet, the frequency of mutation at the lower dosages, i.e., along the ascending portion of the curve, was significantly increased, the peak of the mutation curve was reached at lower energy values, and the decline in

mutation rate at the higher dosages was considerably steeper than that characteristic of the ultraviolet controls (table 1 and fig. 1). These data, which form the experimental evidence for the conclusions drawn by Swanson and Goodgal ('48), indicate that, under the experimental conditions to which the spores were exposed, the mutability of the genes is not simply a function of the amount of radiant energy absorbed, and that the selective killing of mutants by ultraviolet can be greatly enhanced by the action of nitrogen mustard.

TABLE 1

The effect of a pretreatment with nitrogen mustard (0.1% for 30 minutes) on ultraviolet-induced mutation rate in Aspergillus terreus

TREATMENT	ULTRAVIOLET		MUSTARD AND ULTRAVIOLET	
	No. isolates	% mutation	No. isolates	% mutation
(minutes)[a]				
2	290	2.1 ± 0.8	282	4.3 ± 1.2
3	298	4.7 ± 1.2	290	11.0 ± 1.8
5	345	15.9 ± 2.0	275	28.7 ± 2.7
6	170	29.4 ± 3.5	234	38.5 ± 3.2
7	190	30.5 ± 3.3	404	40.7 ± 2.4
9	450	36.4 ± 2.3	344	25.0 ± 2.4
12	141	25.5 ± 3.7	308	13.6 ± 2.0
15	84	23.8 ± 4.7	286	7.0 ± 1.5
18	134	21.0 ± 3.5	284	11.2 ± 1.8

[a] Ultraviolet given at an intensity of approximately 130 ergs/cm²/sec.

These data have been confirmed and extended by a similar study in *Neurospora crassa*, with the frequency of biochemical mutations as well as morphological mutations being considered (Swanson, McElroy, and Miller, '49). The ultraviolet curves for morphological mutations, with and without a nitrogen mustard pretreatment, agree with those in figure 1 except that, in *Neurospora*, the two peaks fell at the same dosage level rather than at the somewhat lower dosages in *Aspergillus* when the mustard was given as a pretreatment. A similar increase in biochemical mutations was found with the nitrogen mustard pretreatment. The frequency of biochemical muta-

tions, however, showed no decline at the higher doses, a not unexpected situation if it is assumed that the biochemical mutations on a complete medium are essentially wild type, and therefore at no selective disadvantage in terms of survival.

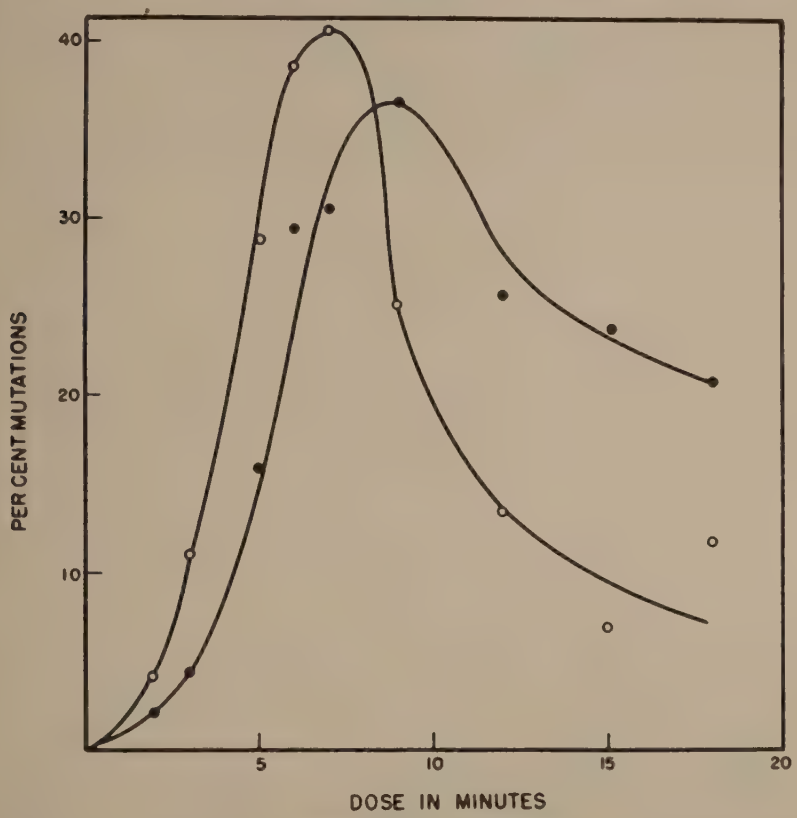

Fig. 1 The relationship between mutation frequency and ultraviolet dosage, with and without a pretreatment with 0.1% nitrogen mustard.
● =Ultraviolet (130 ergs/cm²/sec.).
○ = Mustard + ultraviolet.

ULTRAVIOLET X-RAY COMBINATION STUDIES

Since the previously described experiments have indicated that the mutagenic effectiveness of X rays and ultraviolet can be modified by supplementary nonmutagenic agents, and since it has been shown that ultraviolet exerts an inhibitory action

on the realization of X-ray-induced chromosomal aberrations (Swanson, '44; Kaufmann and Hollaender, '46), it was of some interest to determine the effect of their interaction on mutation rate.

In the initial experiments the dosages of ultraviolet and X rays were kept constant, with only the sequence of radiations being varied. The data are given in table 2. Regardless of whether the ultraviolet or X rays were given first, frequencies of mutation were obtained which were significantly higher than the frequencies expected on the basis that there was no

TABLE 2

Effect of combined radiations on mutation rate in Aspergillus terreus

(Data in per cent)

INDIVIDUAL TREATMENTS		COMBINED TREATMENTS			
		X ray + ultraviolet		Ultraviolet + X ray	
Ultraviolet [a]	X ray [b]	Observed	Expected	Observed	Expected
21.3 ± 1.19	19.7 ± 1.01	42.8 ± 1.27	36.8 ± 1.24 [c]		
15.9 ± 1.17	21.6 ± 1.14			40.0 ± 1.48	34.1 ± 1.43 [d]

[a] Ultraviolet = 5-minute treatment at an intensity of 130 ergs/cm²/sec.

[b] X ray = approximately 50,000 r units delivered over period of 1 hour at 80 kv and 10 ma.

[c] Difference between means: 3.37 times S. E. difference.

[d] Difference between means: 2.7 times S. E. difference.

synergistic action of one radiation on the other, and that the two radiations would give simply an accumulative effect.

In the above experiment, the dosage of ultraviolet was low in order to avoid the depressant effect of higher doses on the rate of recoverable mutants, a factor which would complicate the situation. However, when the dosage of X rays was kept constant, and the dosage of ultraviolet, given as a posttreatment, was varied, a similar trend was found for all treatments (table 3). The observed rate was always higher than the expected. The 5- and 10-minute combination treatments were significantly different from the expected values, P being < 0.01, while the P values for the 15- and 20-minute treatments

lie between 0.05 and 0.01. The combined X-square value, computed by means of a four-place contingency table, was highly significant.

TABLE 3

Effect of combined radiations in Aspergillus terreus

TREATMENT	NO. ISOLATES	PER CENT MUTATIONS	
		Observed	Expected
X-ray [a]	616	9.09	...
UV-5 [b]	598	9.03	...
X-ray + UV-5	532	22.5	17.3
UV-10	614	27.4	...
X-ray + UV-10	610	39.3	34.0
UV-15	400	19.0	...
X-ray + UV-15	619	28.2	24.66
UV-20	614	15.0	....
X-ray + UV-20	420	27.4	22.9
	$X^2 = 25.65$	$N = 3$	$P < 0.01$

[a] X-ray = approximately 25,000 r units delivered over period of 1 hour, at 80 kv and 5 ma.

[b] UV-5 = 5-minute treatment at an intensity of 130 ergs/cm²/sec.

DISCUSSION

Several rather obvious conclusions can be drawn from the data which have been presented. The first conclusion is that it is possible to modify the frequency of mutations induced by a given dose of ionizing or photochemical radiation through the use of supplementary agents, or, to state it differently, it has been demonstrated that the frequency of mutations obtained by exposure of cells to a mutagenic radiation is not necessarily a function of the amount of only the radiant energy absorbed. Thus infrared (Swanson, Hollaender, and Kaufmann, '48) and low temperatures (Medvedev, '35; King, '47) increase the frequency of X-ray-induced mutations, while radiation *in vacuo* has the opposite effect (Hayden and Smith, '49). Kaufmann and Gay ('47), on the other hand, have shown that infrared does not raise the rate of recessive lethals in *D. melanogaster*. Nitrogen mustard effectively increases the frequency of mor-

phological mutations in *A. terreus* (Swanson and Goodgal, '48; also table 1 and fig. 1 in this report) and of morphological and biochemical mutations in *N crassa* (Swanson, McElroy, and Miller, '49).

The second conclusion that can be drawn is that supplementary treatments may affect the rate of survival of ultraviolet-induced mutations. The phenomenon is only detectable, and perhaps only operative, at dosages of ultraviolet higher than that which determines the peak of the mutation curve. Thus, infrared increases the rate of survival without, at the same time, affecting the mutation induction process (fig. 2, Swanson, Hollaender, and Kaufmann, '49), while nitrogen mustard decreases the rate of survival at the same time that it increases the efficiency of the mutation induction process (fig. 1).

The third conclusion serves to reemphasize the difference in mutagenic action of X rays and ultraviolet. That is, supplementary agents do not affect the X-ray- and the ultraviolet-induced mutation frequencies similarly. The infrared studies bear this out, and the nitrogen mustards appear to affect differentially the two types of frequencies. Preliminary experiments carried out in these laboratories indicate that mustard pretreatment of *Aspergillus* spores does not affect the X-ray-induced frequency of mutations. Evidence has already been presented that the mustards materially affect the ultraviolet-induced rate. Stadler ('39, '41), of course, has already pointed out the dissimilarity of effect of the two types of radiation in maize.

The fourth, and last, conclusion is that a supplementary treatment which modifies the induced rate of chromosome rearrangements may or may not similarly alter the mutation rate. Low temperatures increase both chromosome rearrangements (Mickey, '38; Sax and Enzmann, '39) and mutation rate (Medvedev, '35; King, '47). Infrared increases the frequency of chromosome rearrangements (Kaufmann and Hollaender, '46; Swanson and Hollaender, '46; Swanson, '49), and may (Swanson, Hollaender, and Kaufmann, '48) or may not (Kaufmann and Gay, '47) increase the mutation rate. No published data

are available on the effects of nitrogen mustard as a supplementary agent on induced rearrangements by radiations, or similar information on an infrared-ultraviolet combination effect. Most interesting are the ultraviolet-X-ray combination studies. As indicated earlier, the frequency of mutations obtained when the two radiations are employed in sequence is higher than expected if the two radiations are assumed to act independently of each other. However, evidence from both *Tradescantia* and *Drosophila* clearly indicates that ultraviolet exerts an inhibitory effect on the realization of chromosomal rearrangements (Swanson, '44; Kaufmann, Hollaender, and Gay, '47).

Each of the above conclusions suggests ramifications which could be speculatively discussed but this would be unprofitable at the present time. It is obvious that more data are needed concerning the interaction of various agents, mutagenic and nonmutagenic, and their physical mechanism of action, for these points have a bearing on the question of whether the process of mutation induction is a direct or an indirect one. Another question may, however, be raised. Have these data demonstrated that the stability of the gene to mutagenic radiations has been altered by the supplementary agents? Or, to phrase it otherwise, are the modifications in mutation frequency due to increases or decreases in point mutations (intragenic changes) or to increase or decrease in those chromosomal rearrangements which have a phenotypic expression (extragenic changes)? No clear-cut answer can be given, since in the fungi the chromosomes cannot be as minutely studied as in *Drosophila*. The X-ray studies are difficult to interpret because most of what is known of mutation rates as a function of dosage is based on information derived from studies of recessive lethals, and these comprised a heterogeneous lot, some associated with chromosomal rearrangements, some not, and some reversible, others not (for review, see Lea, '46). Herskovitz' ('46) calculations suggest that all recessive lethals are associated with chromsome breakage. On this basis, the increases found by Medvedev and King must be interpreted to

mean that low temperatures raise the rate of primary breakage, and not simply the rate of subsequent recombination.

Some support for the hypothesis that a modification of genic stability has been achieved can be derived from ultraviolet data presented here. Ultraviolet predominantly produces terminal deficiencies (Stadler, '39, '41; Swanson, '40). As Stadler has indicated, ultraviolet, although difficult to compare directly with X rays, produces a high frequency of mutations with little if any effect on the occurrence of rearrangements, while X-ray-induced mutants are generally associated with rearrangements which give rise to pollen defects and low transmission. In haploid organisms such as *Aspergillus* and *Neurospora,* it is highly unlikely that terminal deficiencies would survive and, since ultraviolet produces few internal deficiencies or rearrangements, the morphological and biochemical mutations are in all probability true point mutations. This is particularly true for the biochemical mutations. These are as homogeneous a group of mutants as it is possible to obtain. Being essentially wild type when grown on a complete medium, with no associated morphological change, and reversible for the most part (Ryan, '46; Tatum, '46; Giles, '48), biochemical mutations can scarcely be defined as mutants of the chromosomal deficiency type. The fact that they too can be increased in frequency by supplementary treatments (Swanson, McElroy, and Miller, '49) is the most convincing evidence that the mutability of single genes to a particular dosage of radiation can be altered. This, together with other data discussed above, lends support to the hypothesis that the process of mutation induction is an indirect one, with additional factors besides the absorption of radiation determining the final mutation frequency.

SUMMARY

The data presented in this report indicate that through the use of supplementary treatments the mutation frequencies induced by X rays and ultraviolet can be significantly modified. The significance of these data as they relate to the process of

mutation induction is discussed. It is suggested that, insofar as the ultraviolet-induced mutation rate is altered by nitrogen mustard pretreatment, the increased frequency of mutations results from an increased frequency of intragenic rather than extragenic (chromosomal) changes. Such an interpretation is less certain when modifications in the X-ray-induced mutation rate are achieved.

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THE INDIRECT ACTION OF IONIZING RADIATIONS ON AQUEOUS SOLUTIONS AND ITS DEPENDENCE ON THE CHEMICAL STRUCTURE OF THE SUBSTRATE

WALTER M. DALE
Christie Hospital and Holt Radium Institute, Manchester, England

FOUR FIGURES

I think it is an opportune moment to give you a survey of our more recent, including the latest, experiments on the mode of action of ionizing radiations and the specificity of their effects; because, as you will see in the course of this paper, this problem has reached a crucial point, where current concepts may need modification in the future. Our experiments have been concerned with biologically active substances, such as enzymes or part of an enzyme, and more recently, with another important group of substances — amino acids and their derivatives.

As a representative of substances of high molecular weight we have chosen the crystalline enzyme, carboxypeptidase (mol. wt. 35,000) and of substances of lower molecular weight, alloxazine adeninedinucleotide (mol. wt. of Ba salt 920) which is the prosthetic group of the enzyme D-amino acid oxidase.

With the help of these substances we have been trying to detect specificities of the effect of X radiation in relation to the molecular structure of various substances.

That radiation can act upon a solute through an intermediate product formed from water as well as by direct ionization is now well established. Perhaps less well supported, though widely accepted, is the idea that this intermediate product is identical with OH radicals and H atoms. We have

tried, however, to interpret our results in terms of the radical concept. When pure water is irradiated the radicals speedily recombine after their formation, but a solute present in the water will react with these radicals and this reaction competes with their recombination. In very dilute solutions this competition of recombining radicals is very marked and becomes negligible only when the solute concentration is increased. Then the ionic yield for the reaction of radicals

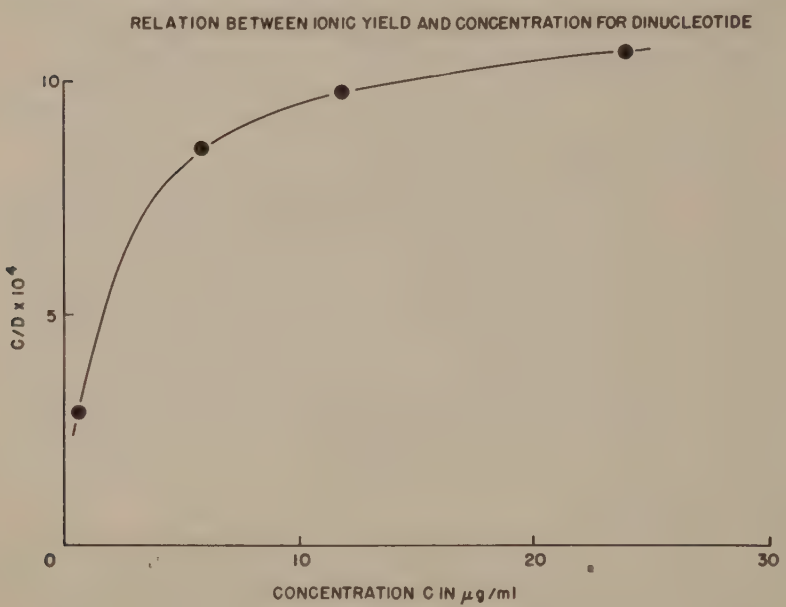


Figure 1

with solute becomes constant. This is shown in figures 1 and 2 for the alloxazine adeninedinucleotide and carboxypeptidase respectively. In the case of carboxypeptidase we were able to observe this constancy of the ionic yield up to a concentration of 15%, a concentration at which carboxypeptidase is no longer soluble. This concentration could only be achieved by centrifuging a suspension of crystals to a solid column.

If now, two or more solutes are present in solution they will compete with each other for radicals and this competition will

lead to a mutual reduction of irradiation effects, i.e., the presence of a second solute will "protect" the first solute which we will call the indicator substance. This protection phenomenon makes it possible to determine indirectly the capability of solutes to react with radicals.

Experiments of this kind consisted of measuring the interference with the radiation effect on the indicator caused by various protective substances added to the indicator solution before irradiation.

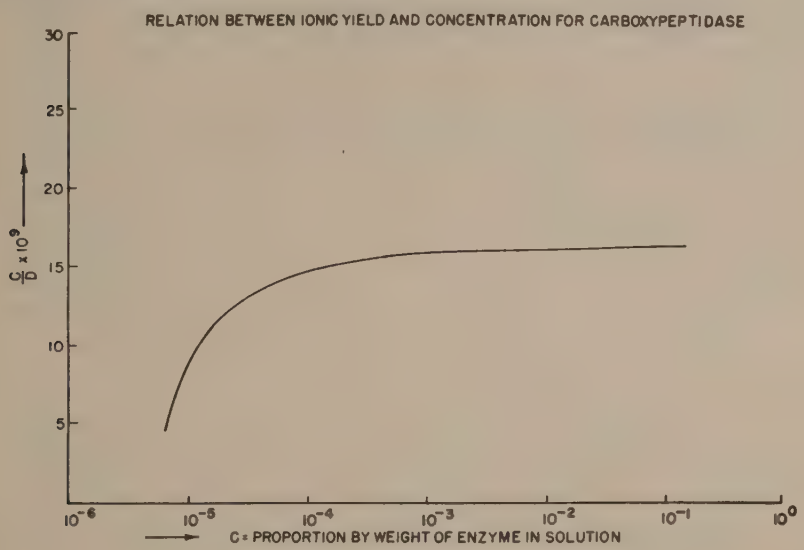


Figure 2

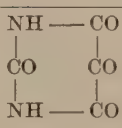
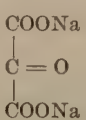
In a preliminary series of experiments (Dale, '47) the amount of the indicator (10 μg carboxypeptidase per milliliter) was kept constant and the amount of protector was also kept at 100 μg per milliliter in most cases. The protective power of added substances was expressed as the following quotient:

$$Q = \frac{D_{(P+E)} - D_E}{P}$$

where $D_{(P+E)}$ and D_E are, respectively, the doses in roentgens required to reduce the indicator activity to 37% of its original

value in the presence and absence of protector, and P the weight of protector per milliliter. These results are shown in table 1. From this table it can be seen that the protective power in the first section of the table is relatively constant, although the molecular weights of the added substances vary

TABLE 1

SUBSTANCE	MOL. WEIGHT	REL. PROTECTIVE POWER PER μG
Tobacco mosaic virus	48×10^6	30
Bushy stunt virus	7.6×10^6	20
Cryst. egg albumin	40,000	17
Denat. egg albumin	..	20
Alanine	89	39
Glucose	180	34
Formate H–COONa	45	320
Oxalate 	88	1.5
Thiourea 	76	~ 1120
Urea 	60	0.5
Alloxan 	142	13
Mesoxalate 	116	7

over a wide range. The protective power, molecule for molecule, therefore appears to be approximately proportional to the molecular weight, which is probably not surprising since the average composition of the more complex organic molecules does not differ greatly. In order to detect specific effects one has to look for special atomic groups and then remarkable differences of the protective power can be found.

Considering such special groups we find the following relaships for the substances listed. If the carbon-carbon bond in oxalic acid is broken and the free valency of the carboxyl group linked to an H atom (thus forming formic acid) the protective power is raised about 200 times. Similarly, if the oxygen atom in urea is replaced by a sulphur atom, thus forming thiourea, a change from a very low protective power to a very high one occurs. If, however, the oxalic acid is modified by the addition of a carbonyl group (mesoxalic acid) or if urea is coupled to mesoxalic acid (alloxan) then the low pro-

TABLE 2

Values of Q for 30 μg/ml of C.P. and for 100 μg/ml of protector

PROTECTOR	Q
Glucose	0.67
Urea	0.03
Dimethylurea	0.35
Thiourea	30.0
Dimethylthiourea	8.8
Egg albumin	0.09

tective power is only slightly raised. Some of these experiments were then repeated, in a number of cases using 30 μg of carboxypeptidase per milliliter which increased the accuracy of the experiments and some further substances added to the list of protectors (Dale, Davies, and Meredith, '49). The results are shown in table 2. The quotient in this table expressing the protective power was changed in order to take the change in indicator concentration into account. Q now equals

$$\frac{D_{(P+E)} - D_E}{D_E} \times \frac{E}{P}$$

where E is the initial concentration of the indicator, the meaning of the other terms of the formula remaining unchanged. The quotient expresses the ratio of the dose taken up by the protector to that taken up by the indicator when unit weights of each are used. Dimethylthiourea and dimethylurea were

examined in order to see the effect of introducing two methyl groups. Dimethylthiourea shows a smaller protective power than thiourea though still appreciable, whereas, dimethylurea is of the order of glucose.

In order to find out more about the chemical reaction of the high protective power of thio compounds, a solution of thiourea was exposed to a high dose of radiation, which caused a precipitation of elemental sulphur. We shall take up this point later on in connection with the kinetics of the radiation effects. The special position of sulphur compounds was further investigated by irradiating colloidal suspensions of sul-

TABLE 3

Relation between protective power and S content of various compounds

	μg P	μS	Q_P	Q_S
Thiourea	5	2.1	55	130
Thiourea	1	0.42	69	166
Dimethylthiourea	5	1.54	18	58
Colloidal sulfur	0.4	0.4	91	91
Colloidal sulfur	1.6	1.6	110	110
$Na_2S_2O_3$	5.2	1.06 (one S)	24	118
		2.12 (two S)	24	59

phur and solutions of sodium thiosulphate. The colloidal sulphur suspension was prepared in these experiments by liberating sulphur from sodium thiosulphate with hydrochloric acid and after appropriate dilution it appears perfectly transparent. Table 3 shows the results of these experiments in which the absolute amounts of the protector (μg P) are given together with the amounts of sulphur (μg S) contained in the compounds and the corresponding quotient (Q_P and Q_S). On inspection of the quotient for comparable amounts of protector substance one has to conclude that: first, all these sulphur compounds have a very high protective power; second, the nonsulphur residue of the molecule plays some part

since the protective power of thiourea is not equalled by any of the other combinations, colloidal sulphur coming nearest to thiourea. In the case of thiosulphate one can be in doubt as to what extent the two sulphur atoms contribute to the protection, the two extremes being that both react equally or one only. Although the preliminary results were qualitatively borne out by the new series of experiments, a new phenomenon was observed. On varying the concentration of the protector it was noticed that in certain cases the value of Q was not constant, contrary to what would be expected of a simple sharing mechanism of radicals by the indicator and the protector. Q declined with carboxypeptidase as indicator in the case of glucose, thiourea, sodium formate, dimethylurea, and dimethylthiourea as the concentrations of these protectors increased, indicating that the protective power was progressively decreasing. This phenomenon which we have called the "changing quotient" (Dale, Davies, and Meredith, '49) occurs at concentrations of the protector and at radiation doses at which the radiation effect is still indirect. Figure 3 shows three examples of it.

In order to explain this phenomenon certain modifications of the current concept of radical reactions seem to be necessary. One has either to assume that the reaction of radicals with protector molecules is of the nature of a chain reaction or that the protector molecule is in some sort of activated state in which it can hand on its energy to the indicator on reverting to its normal state.

We have so far expressed protective power in figures directly derived from experiments; i.e., in terms of X-ray dose and weights of substances employed, without any theoretical assumptions. It seems, however, reasonable to attempt to calculate the protective power; i.e., the capability of reacting with radicals, molecule for molecule, for the various protective substances used.

For this purpose we have modified (Dale, Davies, and Meredith, '49) existing formulas (Lea, '46) for the ordinary sharing mechanism, based on collision frequencies and the

various probability factors in such a way that they now apply for any weight and concentration of the protector and indicator. These modifications consist in introducing, in addition to the lifetime of a radical, terms for the lifetime of the activated protector molecule and the corresponding probabilities of collision and interactions between this new type of molecule and the other solutes. The curves in figure 3 are

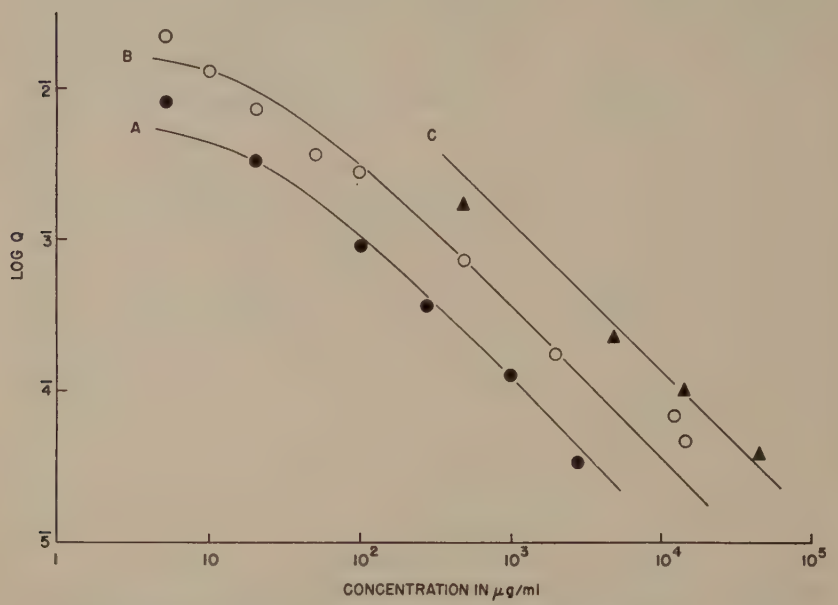


Fig. 3 Changing quotient for C.P. curves are theoretical points experimental.
A (●) — Dimethylurea with C.P. 30 μg/ml
B (○) — Glucose with C.P. 30 μg/ml
C (▲) — Glucose with C.P. 90 μg/ml

theoretical, calculated on this basis, and table 4 summarizes the experimental results calculated on the same basis. It will be seen from column 3 that the ratio of

$$\frac{\text{probability of the various protectors}}{\text{probability of carboxypeptidase}}$$

for reacting with radicals varies over a 10,000-fold range and that one can calculate from the corresponding figures for

alloxazine adeninedinucleotide as indicator (column 4) a similar ratio for the two indicators when sharing radicals. This ratio is an approximately constant figure (column 5) within the limits of experimental error.

DEAMINATION OF AMINO ACIDS AND DERIVATIVES BY X RAYS

Another way of demonstrating the specificity of X-radiation effects can be found in the deamination of amino acids (Dale and Davies, '49; Dale, Davies, and Gilbert, '49). The

TABLE 4

Protective power per molecule of various substances relative to C.P. and D.N.

PROTECTOR	MOLECULAR WEIGHT	P.P. RELATIVE TO C.P.	P.P. RELATIVE TO D.N.	$\frac{(P.P.)_{D.N.}}{(P.P.)_{C.P.}}$
Thiourea	76	4.7	2.1	2.2
Sodium formate	68	3.1	0.74	4.2
Dimethylthiourea	104	1.9	...	..
Glucose	180	0.79	0.21	3.8
Dimethylurea	88	0.4	...	..
Egg albumin	~ 40,000	0.24	0.21	1.1
Alloxan	142	2.1×10^{-2}	...	..
Sodium mesoxalate	152	2.4×10^{-2}	...	..
Sodium oxalate	134	2.0×10^{-3}	...	..
Urea	60	7.5×10^{-4}	...	..
Ratio of values of τ obtained from experiments with indicator only				2.2

P.P. = protective power.

approach to the problem of the relation between radiation effect and molecular structure of the solute in this case differs from that used previously. Whereas then an over-all picture of the radiosensitivity of substances was indirectly obtained by measuring the competition between two solutes for the available radiation energy, there is now only one solute present in any one experiment and the formation of ammonia is directly measured in relation to the molecule from which it is derived. It is therefore not necessary that the radiation reaction progresses beyond changing a few parts per thousand of the initial amino acid concentration for obtaining

measurable ammonia yields, whereas in the case of the inactivation of enzymes, the radiation effect could be measured only in terms of a change of the initial concentration of the solute, a change which had to become fairly large in order to obtain accurate results.

TABLE 5

Ammonia yields from amino acids and other nitrogen-containing compounds in 0.13 M solution after exposure to an X-ray dose of 166,000 r

SUBSTANCE	μG NH_3/ML	SUBSTANCE	μG NH_3/ML
Glycine	8.1	Glycineanhydride	1.3
Glycine HCl	5.0	Glyoxaline	3.0
Glycine NaOH	8.0	Glycylglycine	12.5
α-Alanine	7.2	Glycylglycine HCl	3.8
β-Alanine	4.3	Leucylglycine	6.6
Histidine	11.8	Diglycylglycine	7.0
Histidine HCl	10.6	Proline	0
Lysine HCl	3.7	Urea	0.2
Arginine	7.5	Guanidine carbonate	0
Cystine HCl	6.3 [a]	Thiourea	1.4
Cystine NaOH	19.0 [a]	(Carboxypeptidase,[c] 2.3%, 6.6×10^{-4} *M*	9.1)[a]
Methionine	6.6 [b]		

[a] Large blank.

[b] Relatively large blank.

[c] Mol.wt. 32,000 (Putnam and Neurath, '46); 34,000 (Dale, Gray, and Meredith, '49).

THE SPECIFICITY OF THE DEAMINATION OF AMINO ACIDS AND OTHER N-CONTAINING COMPOUNDS

The specificities of the deamination by X rays is shown in table 5 in which all compounds were irradiated in 0.13 *M* solutions (equivalent to 10 mg of glycine per milliliter) with an X-ray dose of 166,000 r.

It will be seen that the α-amino acids, glycine, alanine, and arginine, are deaminated to nearly the same extent, whereas histidine exceeds the average value. This larger value for histidine may either be due to a contribution from the glyoxaline part of the molecule, since glyoxaline itself yields a certain amount of ammonia on irradiation, or to a weakening

of the bond strength of the α-amino group through the vicinity of the glyoxaline, or to both these causes. If the amino group is in the β position (β-alanine) it appears to be more resistant to deamination. In the case of cystine and methionine, deamination takes place, but no further conclusions are drawn since the blank values were too high. When an equivalent amount of sodium hydroxide is added to glycine deamination remains at the normal level; however, a most striking effect on deamination is observed when an equivalent amount of hydrochloric acid is added to the solution of amino acids and polypeptides. The ionic yield then falls by about one-third the value for the isoelectric state. Apparently the vicinity of the undissociated carboxyl group, which in the dissociated state shows a marked resistance to radiation (Dale, '47), affects the bond strength of the amino group. The effect is masked in the case of histidine where the hydrochloric acid combines in part with the basic group of the glyoxaline. The combination of two glycine molecules in ring form (glycine anhydride) considerably reduced the ammonia value and similarly the peptide linkage in leucylglycine and diglycylglycine does not contribute to deamination, but glycylglycine appears to be an exception yielding more ammonia than glycine. The imino group in proline and the amino groups, combined with carbon dioxide in guanidine carbonate or free in urea and thiourea, are either completely inert or very nearly so.

The experiments with crystalline carboxypeptidase, representing a highly purified protein, were disturbed by large blanks and therefore the value of 9.1 μg of NH_3/ml in table 5 is uncertain.

Further qualitative results were obtained with radiation of DL-cysteine hydrochloride, which is not only deaminated but splits off hydrogen sulfide (Dale and Davies, '51), whereas DL-methionine appears to split off mercaptan.

The effect of adding hydrochloric acid seems to be the opposite to the effect obtained by Weizmann, Bergmann, and Hirshberg ('36) when they irradiated amino acids with ultraviolet light. These authors proposed an ionic mechanism

on account of a minimum quantum efficiency for the isoelectric state of the amino acids examined, whereas in our case, the isoelectric state represents the maximum for the deamination by X rays, addition of sodium hydroxide leaving the maximal value unaltered.

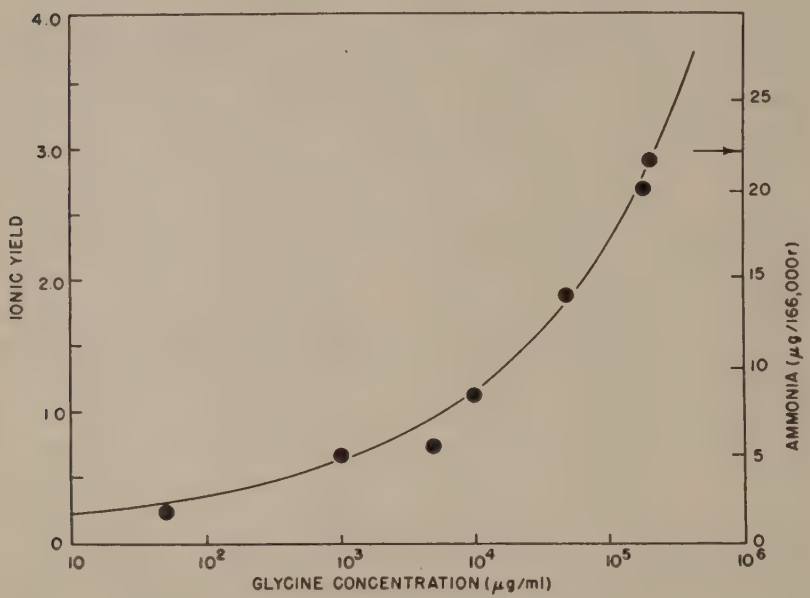


Fig. 4 Ionic and absolute ammonia yield from irradiation of glycine by 166,000 r. Points — experimental; curve — theoretical; arrow — dry glycine. (W. M. Dale, J. V. Davies, and C. W. Gilbert, '49. The kinetics and specificities of deamination of nitrogenous compounds by X radiation. Biochem. J., *45:* 94.)

IONIC YIELD OF THE DEAMINATION OF GLYCINE BY X RAYS

In figure 4 the result of the irradiation of various concentrations of glycine by a dose of 166,000 r is shown and it can be seen that this ionic yield rises to a value far above unity in a 20% solution, although the effect at this concentration is still preponderantly indirect. We have therefore to discuss these experimental results in terms of indirect action, the most characteristic feature of which has hitherto

been the constancy of the ionic yield when the concentrations of the solute are varied, except at extreme dilutions. The deamination of glycine shows no such constancy over the whole range of concentrations, from very dilute to nearly saturated solutions, indicating a more complicated mechanism. As mentioned at the beginning, the usual explanation for the falling ionic yield at extreme dilutions is the recombination of OH radicals and H atoms formed from the water. This explanation may hold for deamination if recombination is still appreciable at the high concentrations because of a possible low affinity of the radicals for glycine (Dale, '43). The experiments show that the ionic yield rises to a value of 2.9 without any tendency to approach a limit and this value is identical with the value obtained for dry glycine. The ionic yields so far observed for biological substances lie between the limits of 0.1 and 1 and therefore the deamination of glycine by X rays seems to be the first example of a nonpolymerizing reaction of an organic compound which can considerably exceed unity. The second example is the liberation of sulfur from thiourea by irradiation (Dale and Davies, '49), which follows a course similar to that of glycine, when its concentration is raised to near saturation point (8% solution).

In order to explain the high ionic yield for deamination, we suggest two alternative mechanisms (Dale, Davies, and Gilbert, '49), one invoking the formation of several pairs of radicals per ion pair and the other a chain reaction. It is obvious that the mechanisms to be discussed for the aqueous solutions will not explain the yield from the dry glycine. We shall not attempt to specify the way the energy is utilized in this case.

To begin with the nonchain radical mechanism, Weiss ('44) has suggested that the effects of ionizing radiations are due to H and OH radicals formed by the splitting of water molecules. It has frequently been assumed that about one effective radical pair is formed for each ion pair (Lea, '46). On the basis of this assumption an ionic yield exceeding unity could not primarily occur, since one radical by its very nature can-

not react with more than one molecule of glycine. However, radicals may be formed by excitation or direct dissociation as well as by neutralization of charged ions (Miller, '48). Miller suggests that, in the case of his inorganic solute, a total of about three pairs of radicals are formed per ion pair (or, more exactly, 32.5 ev of energy absorbed from the radiation). This is energetically possible since it requires 5 ev to form a pair of radicals, and from the 32.5 ev absorbed only about 12 ev are needed to ionize the water molecules. If both radicals are effective (Dale, '47) in reacting with the solute either directly or indirectly by a mechanism such as suggested by Miller, then an ionic yield as high as 6 could be obtained without any form of chain reaction.

The other alternative is a chain reaction. Dainton ('48) has recently shown that OH and H radicals catalyze the polymerization of acrylonitrile and methylacrylonitrile. In this reaction the radical is initiating a chain reaction which can continue without further participation of a radical of the same type. For the deamination of glycine however, it is unlikely that after an OH radical has collided with a glycine molecule, an ammonia formation could progress without further radicals taking part. One might suggest a mechanism shown in equations 1 and 2. All these experiments were done

$$OH + {}^{+}NH_3 \cdot CH_2 \cdot CO\bar{O} = OH \cdot CH_2 \cdot CO\bar{O} + NH^{+}_3 \qquad (1)$$

$$NH^{+}_3 + H_2O = NH^{+}_4 + OH \qquad (2)$$

in ordinary air-saturated solutions, but we have done control experiments in absence of oxygen which show no significant change in ammonia yield. Also the addition of hydrogen peroxide at a concentration equivalent to the amount which could be formed by a dose of 166,000 r (assuming an ionic yield of unity for the formation of hydrogen peroxide) has no effect on deamination.

In our latest experiments performed with DL-serine, we find a course of reaction very similar to glycine. The only difference is that the ammonia yield for serine is considerably greater than for glycine, reaching an ionic yield of 4.6 in

TABLE 6

Deamination of glycine by α-particles

GLYCINE CONCENTRATION μg/ml CELL POSITION	BLOCK 1 200,000			BLOCK 2 200,000			BLOCK 3 10,000		
	A_1	B_1	C_1	A_2	B_2	C_2	α	β	γ
Boron concentration molar	2.25	0	1.13	2.25	0	1.13	2.25	0	1.13
Potassium concentration molar	2.25	2.96	2.60	2.25	2.96	2.60	2.25	2.96	2.60
Ammonia yield μg/ml	800	73	539	968	65	462	312	26	180
Blank yield μg/ml	12	3	12	12	3	12	1	1	2
Ammonia yield less blank μg/ml	788	70	527	956	62	450	311	25	178
Ammonia yield due to α μg/ml	718		457	894		388	286		153
Molecules of NH_3/ml $\times 10^{-19}$	2.57		1.64	3.20		1.39	1.02		0.55
Potassium activity [a] $\times 10^{-5}$	1.36	1.64	1.31	1.20	1.35	1.17	1.48	1.74	1.48
Neutron flux $\times 10^{-10}$	1.02	1.24	0.99	0.90	1.02	0.89	1.12	1.31	1.12
Ion pairs/ml $\times 10^{-19}$	5.64		2.76	4.99		2.47	6.20		3.10
Ionic yield for α radiation	0.46		0.59	0.64		0.56	0.16_5		0.17_7
Mean ionic yield			0.56					0.17_1	
Ionic yield for X radiation			2.89					1.13	
Ratio $\frac{\alpha \text{ yield}}{\text{X yield}}$			19.5%					15.2%	

[a] Potassium activity is the counts/min./ml per molar K 58 hours after the end of the irradiation.

a near saturated solution (5%). Again the value of the deamination for dry DL-serine is near to the value obtained in the almost saturated solutions, i.e., approximately 5.

The two mechanisms suggested could account for an ionic yield exceeding unity but they do not explain the rise of the curve with concentration, although, as I mentioned before, a low affinity of glycine to radicals would extend the region (in which competition by recombination of radicals plays a part) to much higher concentrations. This explanation meets with difficulties when we consider that the liberation of sulfur from thiourea follows a similar course to the deamination of glycine. In the case of thiourea we cannot suppose a low affinity for radicals since in all our experiments thiourea has proved to be a substance of very high protective power.

THE DEAMINATION OF GLYCINE BY α-PARTICLES

Lastly, I should like to mention briefly an experiment in which we compared the efficiency of X radiation and α radiation for the deamination of glycine, the α radiation being obtained by exposing a mixture of glycine solutions with potassium metaborate to a neutron flux. The results are summarized in table 6.

A similar comparison was done in the case of the enzyme carboxypeptidase (Dale, Gray, and Meredith, '49) whereby the α radiation was obtained from radon. It is worth noting that the $\frac{\alpha}{x}$ yield in the case of carboxypeptidase was between 0.05 and 0.1, whereas, in the case of the deamination, it is twice as much. Experiments which are now in progress will decide whether this difference is due to the deaminating action of hydrogen peroxide.

ACKNOWLEDGMENT

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THE ENERGY TRANSFER FROM IONIZING PARTICLES TO AN AQUEOUS MEDIUM AND ITS BEARING ON THE INTERPRETATION OF RADIOCHEMICAL AND RADIOBIOLOGICAL CHANGE

L. H. GRAY

Radiotherapeutic Research Unit, Hammersmith Hospital, London, England

SIX FIGURES

THE DISSIPATION OF ENERGY BY IONIZING PARTICLES

Half a century of research in the fields of radioactivity and electrical discharge in gases has provided fairly detailed information concerning phenomena associated with the passage of ionizing particles through gases. The experimental investigation of the dissipation of energy by ionizing particles in liquids, even in nonconducting liquids, is a matter of very great technical difficulty. It therefore happens that we have only the most meager information regarding energy dissipation in water and aqueous media and we can at best only make a guess as to what is taking place within an irradiated cell by analogy with the phenomena known to take place in gases. This procedure has nevertheless proved useful in the interpretation of radiobiological results.

When living tissue is exposed to 1 r of X or γ radiation, the secondary electrons dissipate energy in the tissue to the extent of approximately 93 ergs/g, i.e., an amount which raises the temperature of the tissue by about 2.5×10^{-6}°C. The increase of energy per unit mass of tissue exposed to 1 rep is slightly less than per unit mass of tissue exposed to 1 r and is about 83 ergs/g. We do not know at all precisely to how many ions this gives rise in water or aqueous media. Such

evidence as we have, mainly derived from radiochemical studies, suggests that the mean energy expended in producing a pair of ions in water is probably nearer 28 volts/ion than the value 32.5 volts/ion determined experimentally for air ionized by fast electrons, and that in consequence rather more than 2×10^{12} ions are formed per gram of water exposed to 1 r. The distribution of the energy absorbed in water between ionization, excitation, and thermal energy is also unknown, but may be presumed to bear a general resemblance to that in gases at normal pressures. The circumstance that radiation doses are measured by ionization methods may, therefore, focus our attention too sharply on the ions as a starting point of the radiobiological changes induced by ionizing radiations. Ionization and excitation are produced in much the same proportion by all types of radiation commonly available for biological studies, so that an estimate of the relative importance of these two modes of energy transfer between the moving particle and the medium cannot be inferred from comparative studies so far made with different ionizing radiations. The balance of energy dissipation does, however, change in favor of excitation with decreasing particle speed, so that information might possibly be gained from studies with epithermal neutrons.

Studies with monochromatic ultraviolet light show that some effects produced by exposure to ionizing radiation can also be produced by the selective absorption of a single photon having a few volts of energy. Point mutations belong to this class of effect. From an analysis of mortality-dose relations, Latarjet has concluded that in certain of the microorganisms which he studied, cell death, which appears to derive from the primary damage produced by a single ionizing particle, may also be brought about by the absorption of a small number of ultraviolet photons within the sensitive region of the cell. Even when the wave length of the ultraviolet light employed is that of maximum biological efficiency, however, the total absorption of energy in the cell needed to produce a given degree of biological damage is much greater when the

organism is exposed to ultraviolet light than when exposed to ionizing radiation. Considering that the total amount of excitation which accompanies ionization will be spread over a large number of modes in many molecules which chance to lie in the path of the ionizing particle, it would seem likely that the energy of excitation is presented to the cell in a form which is less damaging than the most efficient wave length of monochromatic ultraviolet light, and *a fortiori* very much less damaging than the energy of ionization. Monochromatic ultraviolet energy may, however, be used more selectively for certain purposes. It is, for example, more useful than ionizing radiation as a tool for the production of mutations in microorganisms just because it is less lethal than ionizing radiation in relation to its mutagenic potency.

Different ionizing radiations, as, for example, γ rays and neutrons, differ greatly among themselves in the effectiveness with which they induce particular types of damage. From the physical standpoint, it would be surprising if these differences were associated with either the mass of the moving particle or the sign of its charge, since it is very difficult to distinguish physically between the ionization produced by an electron and a proton moving with the same speed. The physically significant parameters are the speed of the particle, and the magnitude of the effective charge which it carries. These determine the rate of transfer of energy to the medium, of which a convenient measure is the linear density of the ions along the track of the particle. Comparative studies covering many diverse types of biological reaction show an almost complete [1] correlation between biological efficiency and linear ion density (Gray, '46, '47, '50). The correlation is an empirical one, which varies in form with the biological

[1] An exception is found in the low efficiency of 8 Å unit X radiation compared with shorter wave lengths in producing chromatid aberrations in *Tradescantia* microspores, despite the greater linear ion density of the less energetic particles. Qualitatively, however, the low efficiency of the 8 Å unit radiation is clearly bound up with the extremely short range of the photoelectrons ($\sim 0.07\,\mu$) which restricts the energy dissipation by any one particle to a region which is too small in relation to the size of the biological structure which is being damaged.

response under observation, and its significance cannot be fully understood without considering the three-dimensional ion distribution along the tracks.

VIRUS INACTIVATION AND GENE MUTATION BY IONIZING RADIATION

It appears, however, that in certain circumstances the linear spacing of the ions may itself be of decisive importance in determining biological efficiency. The inactivation of the smaller viruses and phage particles provides the clearest example of this type of ion density dependence. When the reciprocal of the inactivation dose is plotted against the mean separation of successive primary ionizations, it is seen (fig. 1) that there is a sharp fall in biological efficiency which occurs in the range when the ion spacing is comparable with the size of the virus particle, i.e., when more than one ion cluster is formed in an increasing proportion of the particles, as would be expected if one ion cluster in general represented a sufficient deposition of energy within the particle to cause inactivation (fig. 2).

To Lea's ('47a) thorough and elegant analysis of the experimental observations, I wish to add only one or two remarks. It will be recalled that while good agreement is obtained between the expected and the observed inactivation dose for both low and high ion density radiation in the case of the smallest viruses, the agreement becomes progressively poorer as the size of the virus particle increases if the hypothesis is retained that the whole of the virus is sensitive to radiation. This led Lea to postulate an internal structure of vaccinia virus before it was demonstrated by means of the electron microscope. Lea and Salaman subsequently postulated an inner structure of specially vulnerable material, provisionally likened to the genetic material of higher organisms in the viruses of intermediate size. There thus appears to be an upper limit at about 20 mμ to the size of a living particle which can be inactivated by a single ionization. Genes, which can undergo mutation as a result of individual ionizations are,

from the standpoint of radiation damage, smaller than this, and there seem to be no well-attested cases of a larger volume being inactivated by a single ionization. While there is no reason for rejecting the view that this size represents the dividing line in biological organization, we should perhaps

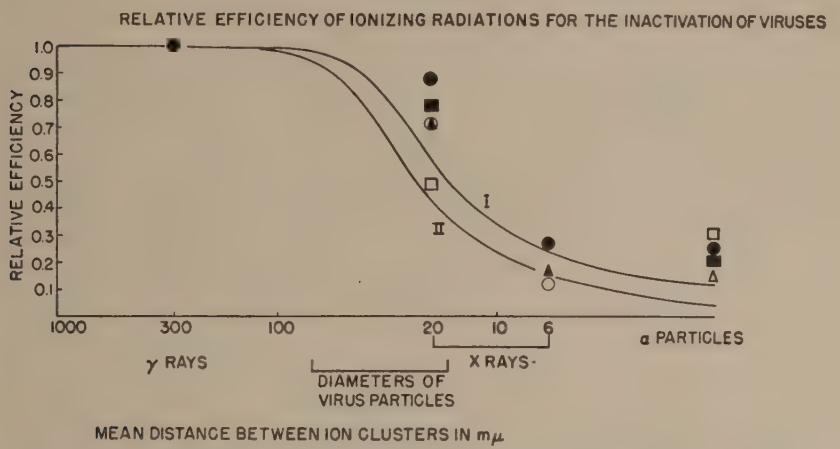

Fig. 1 Relative efficiency of ionizing radiation for the inactivation of viruses. Abscissae = mean distance between ion clusters in mμ. Ion density of radiation increases from left to right. Ordinates = relative efficiency of radiation.

● — Tobacco mosaic virus — (nonspherical)
○ — Tobacco necrosis virus — diameter 25 mμ
▲ — Tomato bushy stunt — diameter 29 mμ
△ — Dysentery phage S-13 — diameter 16 mμ
□ — Coli phage C-36 — diameter 42 mμ
■ — Staphylococcus phage K — diameter 64 mμ

Calculated curves, I for diameter 15 mμ; II for diameter 50 mμ.

The experimental points and the calculated curves are based on inactivation doses given by D. E. Lea, '47 (see Literature Cited).

This figure printed by permission of the British Medical Bulletin from article by L. H. Gray entitled "Comparative Studies of the Biological Effects of X Rays, Neutrons, and Other Ionizing Radiations."

bear in mind that it also happens to be about the mean distance which separates positive and negative ions at the instant of their formation by an ionizing particle, so that only in the case of virus or other particles having dimensions of this order or smaller will the ions of opposite sign be separated by the interphase representing the boundary of the particle.

My second observation relates to the possibility of causing a mutation in a virus particle by ionizing radiations. This has not so far been conclusively demonstrated, though all other

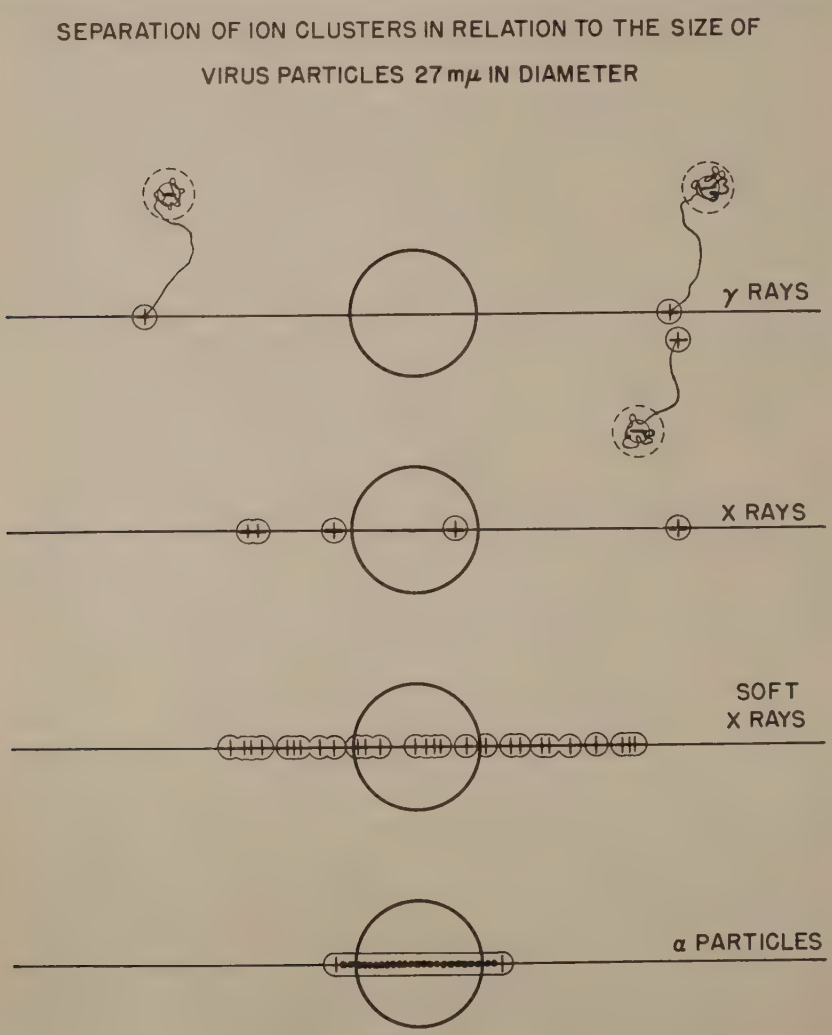

Figure 2

organisms examined have been caused to undergo mutation by exposure to ionizing radiation, and it is clearly of great fundamental interest to discover whether we are here con-

cerned with an intrinsic characteristic of viruses. Mutations in bacteria and higher organisms are associated with single ionizing events, but, for reasons given earlier, an analysis of dose relations cannot distinguish between ionization and excitation as the mutating event. The production of visible mutations requires that the power of the gene to reproduce remains unimpaired. Since the ability of the smallest virus to reproduce is, in general, destroyed by a single ionization, it seems likely that this would be true of genes also; and since ultraviolet light is known to be a mutagenic agent, one is disposed to look to excitation rather than ionization as the cause of visible mutations. No such conclusions can, of course, be drawn regarding lethal mutations since we do not know whether genes undergoing such mutations reproduce in abnormal forms, or fail to reproduce at all. One might conclude, therefore, that ultraviolet light rather than ionizing radiation would be the better tool with which to search for the production of mutations in viruses, but that if an ionizing radiation is used, the radiation of choice will be a low ion density radiation — fast electrons or high voltage X radiation — so as to secure a reasonable chance that the ionizing particle may pass through the organism, causing excitation, but without the formation of any ions within the structure.

Finally, it is of interest to note the apparent insensitivity of the smaller viruses and phage particles to ionization produced in the medium immediately surrounding the particle. When these viruses are exposed to X radiation in the presence of broth, the proportion of virus particles inactivated is in all cases of the same order as the proportion of particles estimated to have been directly ionized, and in a few cases, e.g., the phage S-13, the agreement is very good. Discrepancies are practically all in the sense that fewer particles are inactivated than are ionized. If the phage is sufficiently dilute the degree of inactivation increases, indicating the inactivating effect of ionization produced in the water; but this does not become appreciable till the weight of water present is some ten thousand times that of the virus. This shows the relatively

low effectiveness of ionization produced in the medium but does not formally rule out the possibility that the target presented by the virus includes a sheath of water surrounding the particle. Alpha-ray ionization appears to provide a more critical test, for since X-ray experiments show that a single ion cluster formed within the particle frequently leads to inactivation, it is unlikely that a virus particle can escape inactivation if actually traversed by an α-particle which will produce many ionizations within it. The dense α-ray ionization in the immediate neighborhood of the particle would, therefore, be expected to give the best possible chance of revealing an inactivating effect of the ionized medium, but, in fact, none is found. The targets presented by the small viruses to α-particles are smaller rather than larger than the sizes of the viruses estimated in other ways. We have, however, to bear in mind that despite the very dense ionization along its track, an α-particle may be a less abundant source of radicals for interaction with the solute than the less densely ionizing particles (cf. p. 67).

CHROMOSOME FRAGMENTATION

Turning to chromosome damage, I think we come to a form of radiobiological injury about which it is much more debatable whether the seat of damage is in the biological structure itself or in the surrounding medium. The contrasted dose relations and intensity dependence observed with neutrons and α-particles, on the one hand, and X and γ radiation on the other, as well as the high frequency with which two threads nearly in juxtaposition at the time of irradiation are broken at the same locus, appear to leave no room for doubt as to the effectiveness of the local dissipation of energy. Nevertheless, as Allsopp and Catcheside ('48) have pointed out, this is not incompatible with a chemical mechanism of breakage, since the dose relations referred to only establish that a break occurs as a result of the passage of an ionizing particle either through or near the chromosome thread. There

does, in fact, appear to be definite evidence that the effect of radiation on the medium surrounding the chromosome in the immediate vicinity of the break can profoundly affect the frequency of break production. Kotval and I ('47) were led to this conclusion by the unexpectedly high efficiency which was found when the prophase chromosomes of *Tradescantia* microspores are broken by α radiation. Whether we consider the absolute frequency of break production, the frequency relative to neutron radiation, which it had already been necessary to assume to be nearly at the rate of one break per transit of a proton through the chromosome, or the proportion of breaks produced in the two sister chromatids at the same locus, we were unable to escape the conclusion that something like half the breaks produced by α radiation must be ascribed to energy dissipated in the surrounding medium. The fact that breakage frequency could be affected by the physiological condition of the cell at the time of irradiation points in the same direction. The recent evidence of Thoday and Read ('47, '49) seems to be of special interest in this connection. You will recall that Thoday observed that the number of abnormal anaphase figures resulting from a given dose of X radiation to *Vicia faba* roots was reduced to one-third when the irradiations were carried out under anaerobic conditions. Comparative experiments with α radiation showed some reduction in the breakage frequency, but much less than in the case of X radiation. This is very interesting because, as the authors point out, the difference parallels the relative efficiency with which hydrogen peroxide is produced by the two radiations in gas-free and aerated water. Bonet-Maury and Lefort ('48a, b; '50) found that dissolved oxygen is quite without influence on the hydrogen peroxide yield with α radiation, but markedly influences the X-ray yield. Thoday observed a small but distinct difference in breakage frequency under α radiation when dissolved oxygen is absent. The difference between the influence of dissolved oxygen on the chemical and cytological changes, though small, is interesting and is perhaps understandable when we take δ-ray ionization into

account. We will, however, first consider the influence of δ-ray ionization on radiochemical yield.

BIOCHEMICAL EFFECTS OF RADIATION

The consideration of the chemical aspects of δ-ray ionization arises out of a series of experiments conducted by Dale, Gray, and Meredith ('49) during 1943–45 in which a careful quantitative comparison was made between the effects of X and α radiation on the enzyme carboxypeptidase. The degree of inactivation was found to be an exponential function of the dose, as has been found also to be the case with the sulfhydryl enzymes examined by Barron and his collaborators ('49). The X-ray inactivation dose in the case of carboxypeptidase was found to be inversely proportional to concentration over a wide range of concentrations, indicating that the enzyme was inactivated by interaction with the labile product resulting from the irradiation of the water. The enzyme is not inactivated by the addition of hydrogen peroxide to the solution and, at a concentration of 3×10^{-5} g of enzyme/ml, the X-ray yield is independent of the concentration of dissolved oxygen.

Unlike Barron's enzymes, however, which appeared to be inactivated with something like the same efficiency by X and α radiation, carboxypeptidase shows a very much higher ionic yield with respect to X than to α radiation (fig. 3). In the concentration range 10^{-4} to 10^{-2} the ratio is about 20:1. This ratio may vary a little with concentration (fig. 4) but we were puzzled by the fact that the ratio does not tend to zero but to a finite value at zero concentration. It was argued that if the chance of inactivation between radicals (or whatever the labile product might be) and enzyme was as low as one in 20 (the maximum value compatible with experiment in the case of α radiation) then the probability of interaction, and therefore the yield, should decrease in proportion to the concentration. Gilbert ('49) has since pointed out that this is not true if interaction takes place in an expanding column of radicals, but the thought prompted us to look for an ex-

planation of the finite yield at low dilutions and Meredith suggested that this might be due to the δ rays. Figure 5 shows the δ-ray contribution to the total ionization produced by

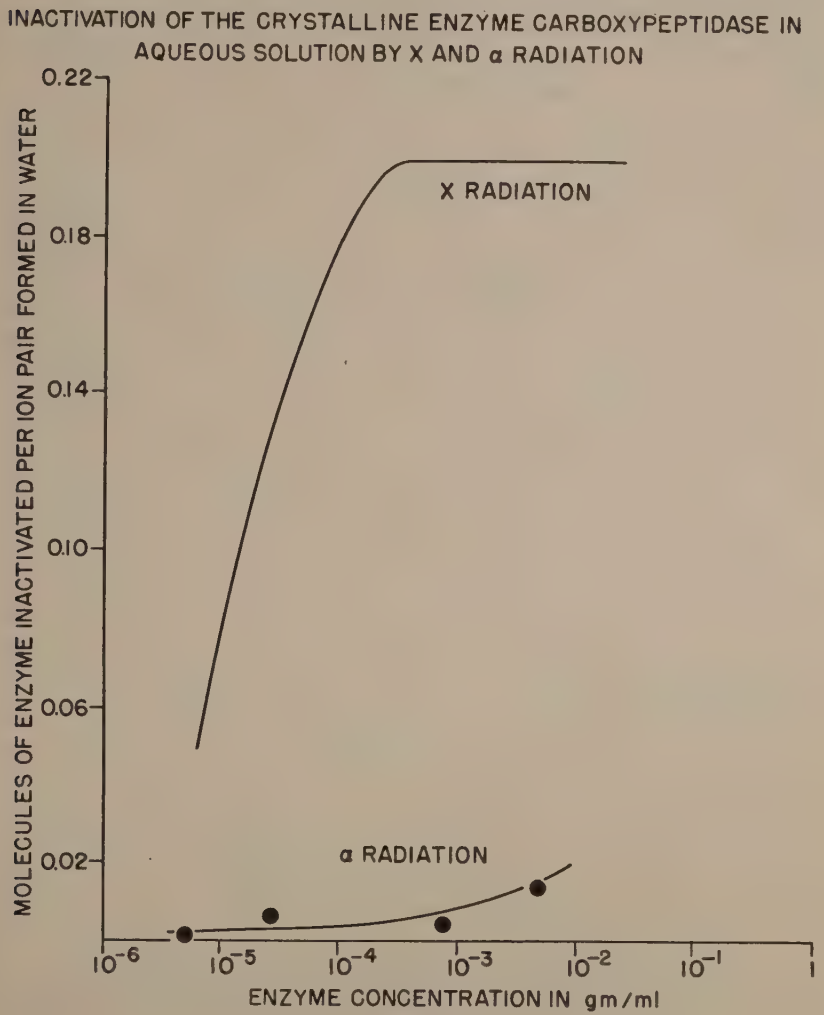

Figure 3

α rays when we consider only that part of the δ-ray track which lies outside the column of α-ray ionization. In view of the uncertainty which exists concerning the radius of the

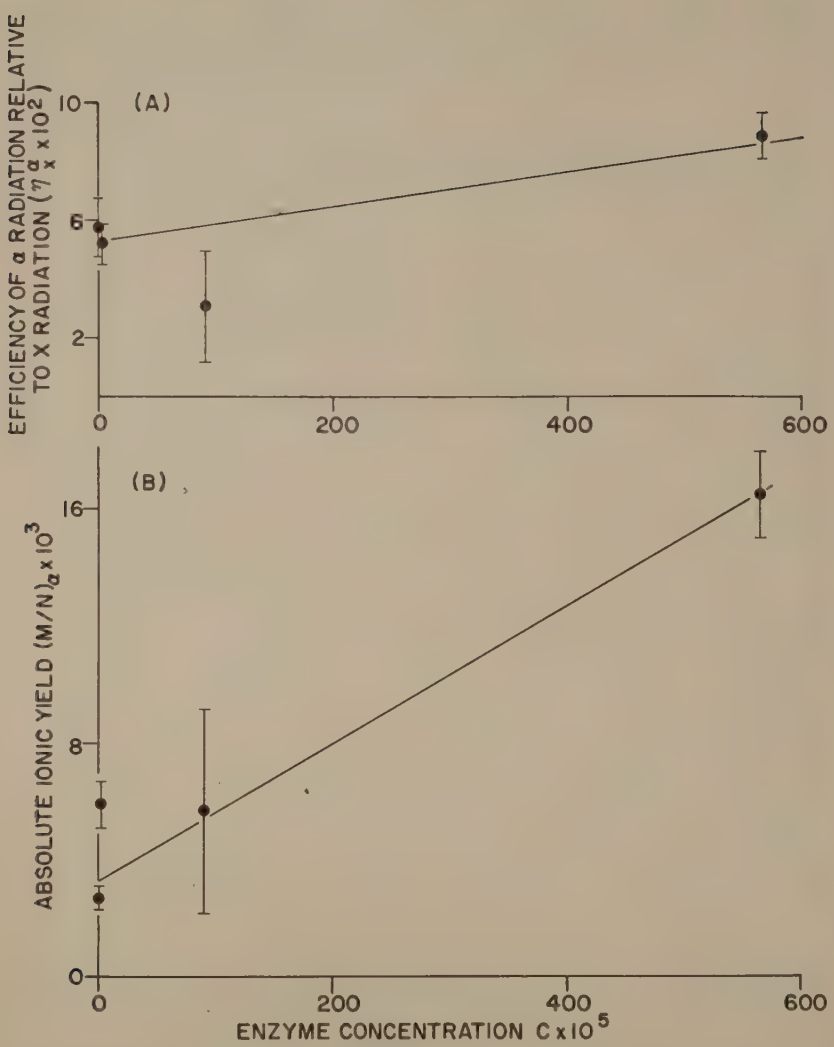

Fig. 4 (a) The inactivation of carboxypeptidase by X and α radiation. Ratio of α- to X-ray ionic yield as a function of enzyme concentration. (b) The inactivation of carboxypeptidase by α radiation. The absolute ionic yield as a function of enzyme concentration.

This figure published by the permission of The Royal Society from an article by W. M. Dale, L. H. Gray, and W. J. Meredith ('49) entitled "The Inactivation of an Enzyme (Carboxypeptidase) by X and α Radiation," Phil. Trans. Roy. Soc., London, A *242:* 33.

negative ion column in water, calculations were carried through for two values of this radius. These gave estimates of 4 and 10% respectively for the effective δ-ray ionization

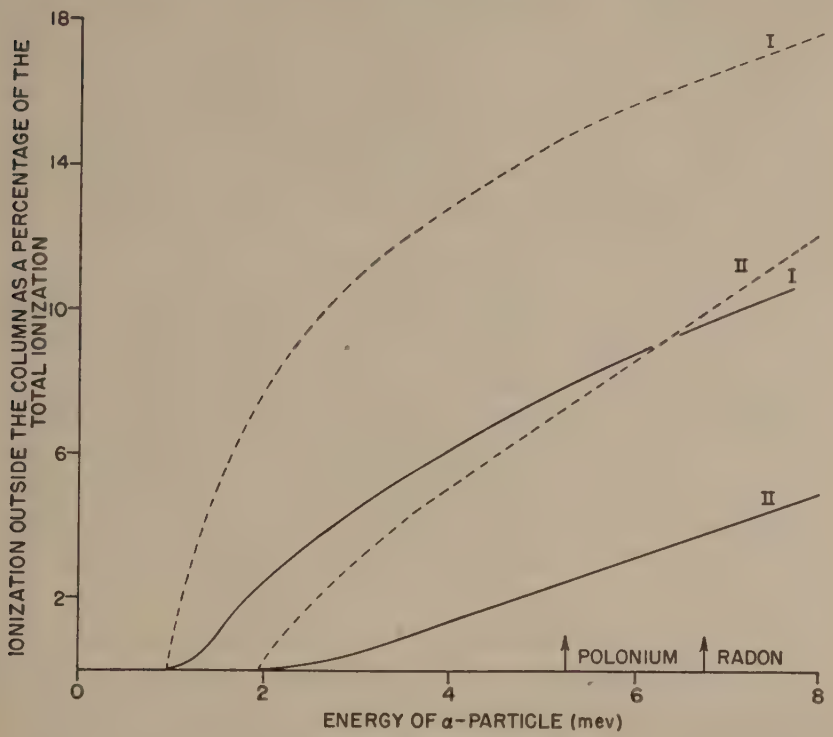

Fig. 5 δ-Ray ionization. The ordinates show the ionization produced by all δ rays at distances greater than an arbitrary distance, d, from their point of origin, expressed as a fraction of the total α-particle ionization. Broken lines, abscissae refer to the instantaneous energy of the α-particle. Solid lines, abscissae refer to the initial energy of the α-particle. In each case curve I refers to $d = 0.015\,\mu$ in water and curve II to $d = 0.04\,\mu$ in water.

This figure is published by permission of The Royal Society of London from an article by W. M. Dale, L. H. Gray, and W. J. Meredith ('49) entitled "The Inactivation of an Enzyme (Carboxypeptidase) by X and α Radiation." Phil. Trans. Roy. Soc., London, A *242:* 33.

associated with radon α-particles, in very satisfactory agreement with the experimentally observed ratio of α-ray to X-ray efficiency at low enzyme concentrations.

The steep fall in the curve (fig. 5) with descending α-particle energy is noteworthy since it implies that, with enzymes which behave toward irradiation in the same manner as carboxypeptidase, an extremely low yield is to be expected from the products of disintegration induced in boron and lithium by slow neutrons.

We went on to consider in more detail the spatial distribution of ions resulting from exposing water to X and α radiation, and arrived at some conclusions which Lea ('47b, and private communication) had reached independently shortly before his death.

The primary positive ions formed by all types of ionizing radiation lie on the geometrical path of the ionizing particle. If we take it that the negative ions are formed by electron attachment at a mean distance of 15 mμ, then the initial disposition of ions formed by fast and slow electrons and α-particles will be as depicted in figure 6.

Read ('49) has pointed out that the separation of positive and negative charges depicted in the α-ray case would be associated with an amount of electrostatic energy which, per ion pair, is large compared with the energy of thermal agitation so that the negative ions will be drawn inward onto the axis of the track and this tendency will oppose diffusion which is usually the only factor considered in the subsequent movement of the ions. While this is undoubtedly true, it will not affect our discussion of chemical effects of ionization if, as is supposed to be the case, the ions undergo virtually instantaneous dissociation into a hydroxyl radical and a hydrogen ion on the one hand, and a hydrogen atom and hydroxyl ion on the other. The radicals will then diffuse outward, and the ions will recombine. This recombination will contribute to the rise in temperature on the axis of the column which might in the case of an α-ray track be a few degrees centigrade, but which will in any case probably be small compared with the temperature rise due to some measure of recombination of radicals.

It is evident from figure 6 that, within the inner sheath in the case of α radiation, there is formed initially an extremely high concentration of hydroxyl radicals ($\sim 3\,M$) and that these radicals will make many collisions in pairs before the column is sufficiently expanded by diffusion to allow the number of collisions between unlike radicals to be comparable with

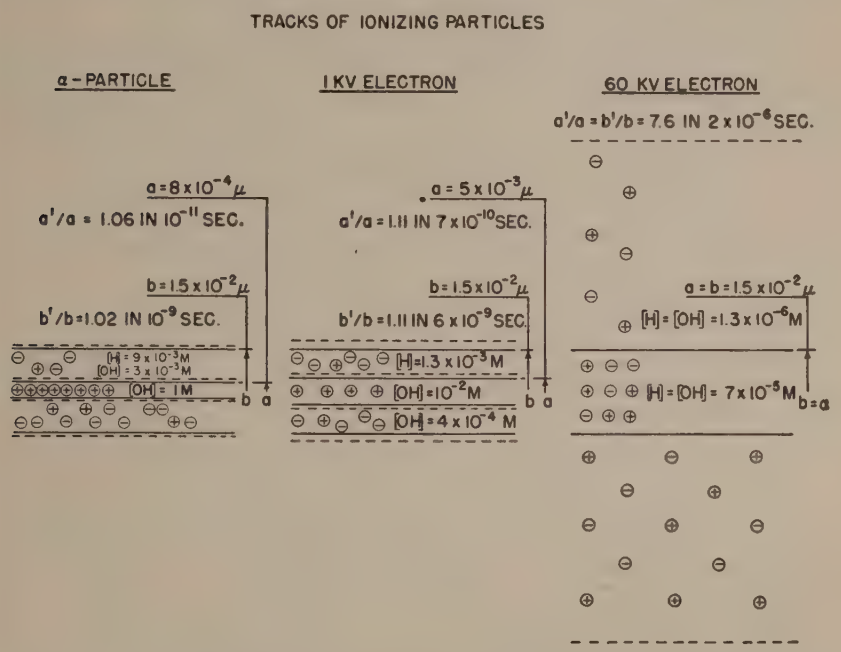

Fig. 6 The dotted lines show the expansion of the columns during the time that 50% of the radicals make one collision. (From "Electrons, Neutrons, and Alpha Particles," by L. H. Gray, in Biophysical Research Methods, F. M. Uber, ed. Copyright, 1950, Interscience Publishers, Inc., New York.)

that between like radicals. We believe that the high initial concentration of OH radicals probably accounts for the well-established experimental fact that the irradiation of water by high ion density radiation, particularly α radiation, gives rise to the production of hydrogen peroxide irrespective of the presence or absence of dissolved oxygen. If the accuracy of the chemical experiments permitted, it might be found that

an additional 5% of hydrogen peroxide would be found to be formed by α radiation in the presence of oxygen on account of the associated δ rays discussed above.

On account of the much lower ion density along the track of a δ ray than along that of the α-particle which generated it, the 5% of δ-ray ionization is spread along a total length of track which, according to Lea's estimates, more than equals the length of the α-particle track itself in the case of a 5-mev particle. All the δ-ray track is probably not of sufficiently high ion density to have a high chromosome breaking efficiency but when due allowance is made for this, I have estimated in another connection that δ rays probably add something like 20% to the effective length of the α-particle track from the point of view of chromosome fragmentation. If this is so, we should expect the removal of dissolved oxygen to decrease the frequency of chromosome fragmentation by α radiation by something like this amount, though the change in hydrogen peroxide production is barely measurable. This accords well with Read and Thoday's observations referred to earlier.

There is one other aspect of the initial spatial distribution of ions along an α-particle track to which I should like to refer, namely, the difference which might be expected between the relative effectiveness of α and X radiation in transforming oxidizing as compared with reducing solutes. Clearly, the combination of hydroxyl radicals in pairs on the axis of the α-ray track would leave the hydrogen atoms free for interaction, by contrast with the case of electron ionization in which recombination of hydrogen and hydroxyl is favored. We may then expect that, while a very high concentration of solute would be expected to be necessary for reaction with hydroxyl radicals under exposure to α radiation, a solute which reacts with hydrogen atoms to give a product which does not react with formed hydrogen peroxide might show an ionic yield at very low concentrations, which is actually higher for α than X radiation.

In conclusion, it should be emphasized that the condition shown in figure 6 only represents an initial condition. The

distribution between the location of positive and negative ions and the radicals to which they give rise rapidly becomes blurred through the outward diffusion of both types of radical. Mutual combination and recombination of radicals, as well as interaction with solute, take place in such rapidly expanding columns, i.e., under conditions of rapidly falling radical concentration and rapidly falling mean energy of activation — a situation which, from the standpoint of the chemical kinetics leading to radiobiological damage, represents a high degree of complexity.

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EXPONENTIAL AND SIGMOID SURVIVAL CURVES RESULTING FROM ALPHA AND X IRRADIATION OF ASPERGILLUS SPORES[1]

RAYMOND E. ZIRKLE, DOROTHY F. MARCHBANK, AND KATHRYN D. KUCK

Institute of Radiobiology and Biophysics, University of Chicago, and Division of Biological and Medical Research, Argonne National Laboratory

THREE FIGURES

Any type of high-energy radiation either consists of or produces fast charged particles (electrons, mesons, or atomic nuclei). By changing the nature of the radiation or the conditions of irradiation in such fashion as to alter the linear energy transfer (LET),[2] the dose-effect curves for many radiobiological actions can be altered (Zirkle, '43). Changes in slope of dose-effect curves are common but changes in shape are rare, and in particular a change from exponential to a

[1] This work was performed under contract N6ori-20 with the Office of Naval Research, in cooperation with the U. S. Atomic Energy Commission.

[2] This is the energy transferred from the fast charged particles, per unit length of their paths, to the biological material in or near these paths. This quantity is frequently termed the space *rate of energy loss*, but this implies interest only in the kinetic energy lost by the particle. It has also been termed the *linear energy absorption* (Zirkle, '40), which implies interest only in the energy gained by the irradiated object. The word *transfer* implies interest in both the particle and the material it traverses. Related terms are *specific ionization* and linear *ion density*, both used to designate the number of ion pairs produced per unit length of the path of the high-energy particle. They are subject to the following objections: (a) Both focus attention on the production of ions, whereas excitation of molecules may also be of radiobiological importance. (b) The ionization per unit path *in tissue* cannot at present be determined, whereas the energy transferred (LET) can be estimated closely enough for most purposes. LET is approximately proportional to specific ionization, the conversion factor for air being 32.5-35 ev per ion pair. The conversion factor for tissue, although not known, is sometimes assumed to be roughly the same.

sigmoid curve has been observed for very few radiobiological actions. Such a change in shape is of interest because it indicates that the radiobiological action in question involves a definite number of decisive events per cell (or other biological unit) (Zirkle, '50).

In 1938 Haskins published some distinctly sigmoid survival curves of electron-irradiated spores of *Aspergillus niger*. On the other hand, Zirkle ('40), using *Aspergillus* cultures supplied by Haskins, found that the survival curves of α-irradiated spores were clearly exponential. In view of the great difference in linear energy transfer from the electrons and the α particles, the corresponding disparity in the shapes of these survival curves appeared to merit a careful check with both radiations in the same laboratory, under identical conditions and with identical biological technique.

EXPERIMENTAL PROCEDURE

Since considerable radiobiological work had been done with *Aspergillus terreus* (e.g., Hollaender and Emmons, '46) it was decided to test this species rather than *A. niger*. Doctor Alexander Hollaender, Oak Ridge National Laboratory, kindly supplied a culture of the strain used by himself and associates, and the stock was maintained on potato-dextrose agar slants at 20–25°C.

For each experiment, several loopfuls of spores were taken from a culture 10–23 days old, placed on a microscope slide and thoroughly mixed. From this mixture each experimental sample, consisting of several thousand spores, was transferred with the tip of a needle to one face either of a lucite disc 1.5 mm thick and 2 mm in diameter or of a lucite block 1.5 mm thick and 3 mm square.[3] In either case the area covered by spores was a circle about 2 mm in diameter. In α-ray experiments care was taken that the spores formed a mono-

[3] This operation is inconvenient because of the tendency of the spores to stick together. Various detergents, notably Triton X, were found to be effective in separating them, but were also toxic in erratic fashion; accordingly they were not used in any experiments reported here.

layer and thus did not shield each other. Before the spores were spread, the lucite discs and squares were sterilized by ultraviolet irradiation.

For X irradiation, a suitable number of spore-bearing lucite blocks were placed at the center of the bottom of a plastic box 10 cm in diameter and 2 mm deep. The sides of the box were of lucite and both top and bottom were of celluloid 0.7 mm thick. When the top was in position, it was about 0.5 mm above the spores. The box was centered close beneath the X-ray tube. The X-ray outfit, a General Electric Maximar, was operated at 250 kv and 15 ma. No filter was used except that inherent in the apparatus. The dose rate, determined with a Victoreen condenser r-meter, varied between 950 and 1000 r/minute on different days. Irradiation was briefly interrupted at proper intervals for removal of samples, graded doses thus being delivered.

The α particles were from polonium uniformly deposited, by distillation, in a layer of negligible thickness over one face of a palladium disc 4 mm in diameter.[4] Two arrangements for irradiation were used. In arrangement A, ten samples on lucite discs were placed in a circle and irradiated simultaneously. The spatial relations between two of the samples and the α source are diagrammed in figure 1 A. In arrangement B, the samples were irradiated one at a time (fig. 1 B). For each arrangement, the α flux (F) at any point P on a spore preparation was calculated from the general relation

$$F = \frac{2.3}{4} n \log \frac{\sqrt{(a^2 + b^2)^2 + 2(a^2 - b^2)r^2 + r^4} + r^2 + a^2 - b^2}{2a^2}, \quad (1)$$

where n is the number of polonium disintegrations per unit time per unit area of the source; r is the radius of the source; a is the distance from the plane of the source to a parallel

[4] The α sources were furnished by the Chemistry Division of Argonne National Laboratory. We are especially indebted to Dr. W. M. Manning and Mr. P. R. Fields for their courtesy and to Mrs. Sylvia Warshaw and Miss Eileen Gerngross for their services in preparing the sources.

plane through P (fig. 1 C); and b is the normal distance from P to the axis of the source. The value of n was determined with a low-geometry α counter by the Chemistry Division of Argonne National Laboratory. With arrangement A, the maximum variation of F over the spore-covered area was about 3%, and with arrangement B about 1%. The dose rate was FE, where E is the energy transferred from each α particle, per unit length of its path, to the biological material. Since the particles traversed the spores (about 5 μ in diameter) while still moving fairly rapidly, E varied only

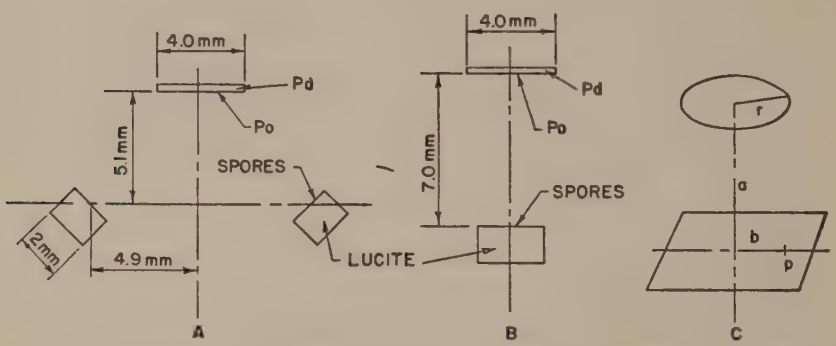

Fig. 1 Geometrical relations between α sources and spores. (a) Section through axis of Arrangement A (2 of the 10 spore samples shown). (b) Section through axis of Arrangement B. (c) Perspective view showing spatial quantities necessary for calculating α flux at any point P in a plane parallel to the source.

slightly across any spore and may be represented by a mean value which we take to be 100 kev/μ (Lea, '47a, table 12). This figure is somewhat uncertain because the stopping power of the spore material is not accurately known (Lea, '47a, pp. 350–352), but this is not particularly serious here, since we are interested primarily in the shapes, rather than the slopes, of survival curves. In converting doses to rep, 1 rep has been set equivalent to 90 ergs/gram, which is the energy absorption from 1 r of 250 kv X rays (Lea, '47a, table 2). No correction has been made for the specific gravity of the spores, since it would be the same for both radiations.) In

each experiment, graded doses were used to obtain data for a fairly complete survival curve.

After either X or α irradiation each lot of spores was transferred, by successive "printings," to the surface of a block of Difco potato-dextrose agar about 15 mm square and 3 mm thick. The various "planted" agar blocks were placed in a petri dish, which contained additional strips of agar to prevent desiccation, and were incubated at 20°C. After a suitable length of incubation, the spores of each lot were examined microscopically and classified as germinated or ungerminated. As in *A. niger* (Zirkle, '40), the normal germination of the spore of *A. terreus* involves two easily distinguishable events: first, a swelling to 4 or 5 times the diameter of the air-dry spore; second, the protrusion of a germ tube from the swollen spherical spore. Any detectable germ tube, however short, was arbitrarily taken as a criterion of "germination," and hence of "survival," for the purposes of this study. (It will be noted that this criterion of survival differs markedly from that of Stapleton, Hollaender, and Martin, as described in their adjacent paper.) Since inhibition of the initial swelling requires many times the doses which inhibit germ tube formation, swelling was used as a criterion of viability of each spore; this was a great convenience, because a large and variable percentage of the air-dry spores do not germinate under our conditions. Accordingly, in each irradiated sample, the percentage of germination (survival) was simply the percentage of spores forming germ tubes among the total spores which had swollen.

Counts were made daily until no more spores germinated. Since irradiation delayed as well as inhibited germination, final counts were usually obtained on the third day after the larger doses and on the second after lower doses. Three or four hundred spores were classified in each lot, except that, when survival was less than 5%, 1200 were classified. Care was taken to classify every swollen spore in each microscope field selected for counting.

RESULTS AND DISCUSSION

Three α-ray experiments were performed as shown in table 1. The three sets of data are plotted on a semi-logarithmic grid in figure 2. (Since the alpha emission of the source used in experiment 3 was not determined, dose is plotted in terms of exposure time.) It will be noted that in each case the experimental points cluster fairly well about a single straight line. Accordingly we may conclude that the α-ray survival curve of the *Aspergillus* spore, with respect to our criterion of survival, is exponential.

Four X-ray experiments were performed; the data are plotted in rectangular coordinates (fig. 3). It is readily seen that none of the 4 sets of experimental points can be fitted

TABLE 1

EXPERIMENT NUMBER	ARRANGEMENT FOR IRRADIATION	DOSE RATE (rep/min)
1	A	350
2	A	330
3	B	Undetermined

by an exponential curve (dotted lines). They can, however, be fitted approximately by a curve described by a simple theoretical expression. If we assume that germ tube formation is inhibited by a mechanism in which a constant small number of identical cellular entities per cell are each inactivated by a small number of decisive radiobiological events (Zirkle, '50), the survival curve is described by the general "hit-theory" formula (Timoféeff-Ressovsky and Zimmer, '47)

$$\frac{N}{N_0} = 1 - \left[1 - e^{-hD} \sum_{k=0}^{m-1} \frac{(hD)^k}{k!} \right]^n \qquad (2)$$

where n is the number of identical cellular entities involved, m is the number of decisive events necessary to alter each entity, D is dose, and h is the probability, per unit dose, of

a single decisive event per cell. When n and m are assigned small integral values, equation (2) yields values of the survival fraction N/N_0 as shown in table 2. In figure 3 the four unbroken curves are drawn according to the two-hit formula, $N/N_0 = 2e^{-hD} - e^{-2hD}$, i.e., on the assumption that one

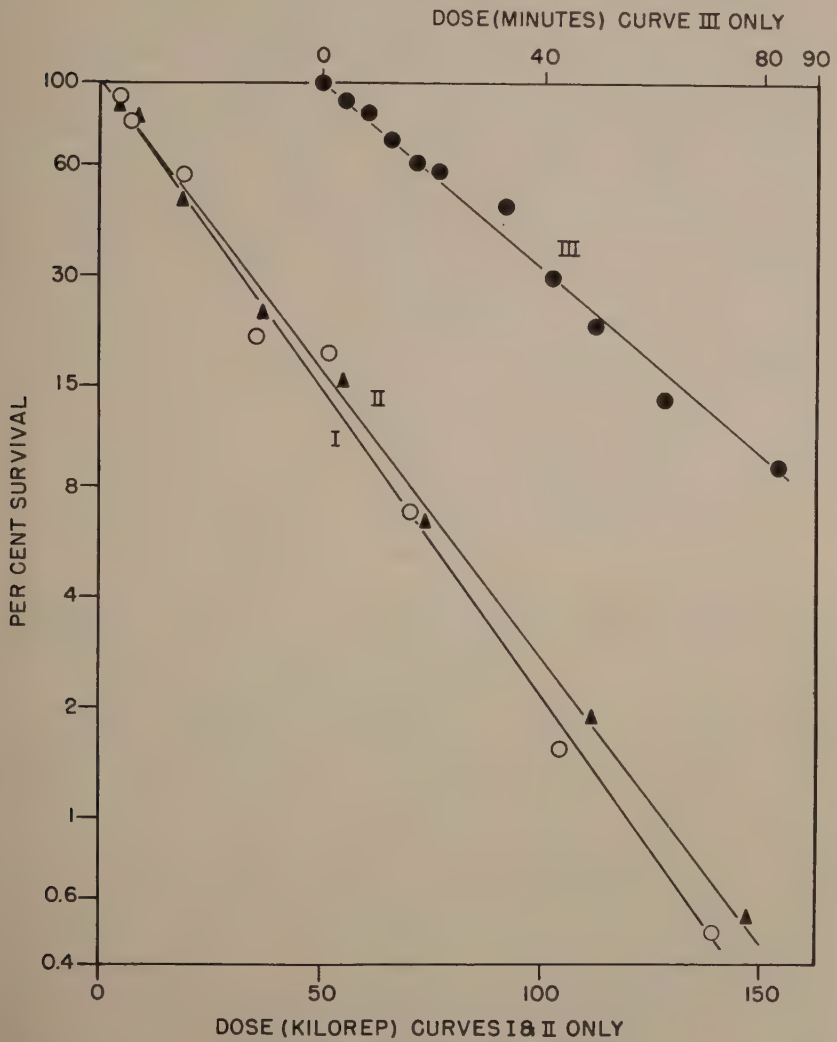

Fig. 2 Relation between α-ray dose and survival fraction of *Aspergillus* spores.

decisive event must occur in each of two distinct cellular entities. Considering the scatter of the experimental points, it will be noted that this formula fits fairly well except in figure 3 B, where, below a survival fraction of 0.3, all experimental points deviate systematically from the theoretical curve. Curves drawn according to the other two-hit formula,

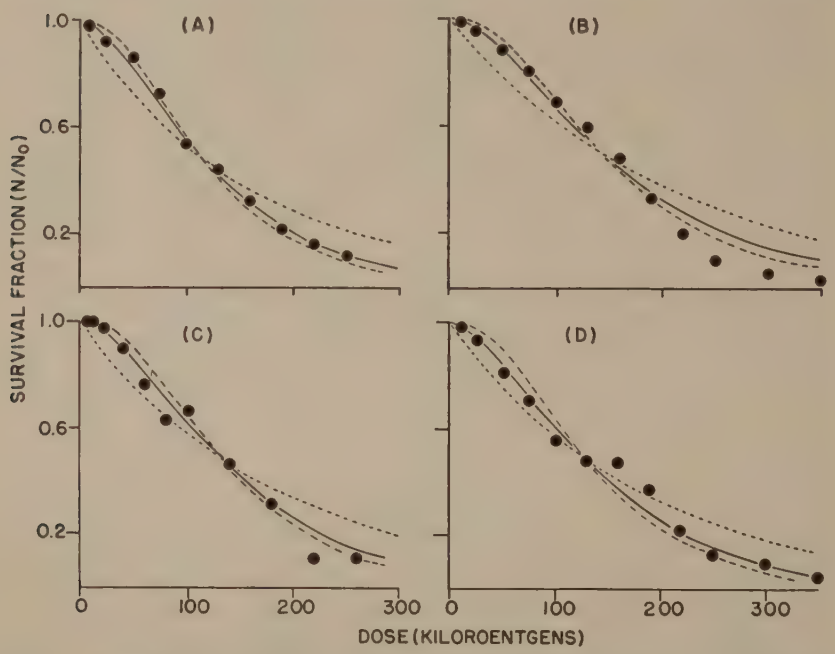

Fig. 3 Relation between X-ray dose and survival fraction of *Aspergillus* spores. Curves drawn according to theoretical formulas: dotted, $N/N_0 = e^{-hD}$; unbroken, $N/N_0 = 2e^{-hD} - e^{-2hD}$; dashed, $N/N_0 = e^{-hD}(3 - 3e^{-hD} + e^{-2hD})$. Values of h adjusted so that all curves intersect at $N/N_0 = 0.5$.

$N/N_0 = e^{-hD}(1 + hD)$, i.e., on the assumption that two events of equal probability must occur in a single cellular entity, fit the points about as well as $N/N_0 = 2e^{-hD} - e^{-2hD}$ but are not shown because they would nearly coincide with the latter when the 50% points are made to coincide. The dashed curves are drawn according to the three-hit formula $N/N_0 = e^{-hD}(3 - 3e^{-hD} - e^{-2hD})$, and it will be noted that,

with the exception of figure 3 B, they deviate much more from the experimental points than do the two-hit (unbroken) curves. Curves according to the other three-hit formula, $N/N_0 = e^{-hD}(1 + hD\,h^2D^2/2)$, are not shown, but would deviate slightly more. Accordingly, considering the results as a whole, it appears that, among the various simple theoretical curves, the two-hit ones are most nearly satisfactory.

Despite the uncertainty regarding the exact theoretical fit of the X-ray survival curves, there can be no doubt that they are of a multiple-hit type, whereas the α-ray curves (fig. 2) are of the one-hit type. This is the one significant result of these experiments, and the main conclusions to be drawn are (a) that, in the radiobiological action which results in inhibition of germ tube formation, the number of entities which must be altered in each individual cell is quite small, and (b) that, whereas all of them are altered by one suitably located α track, two (possibly three) electron tracks are required to produce the same alteration(s).

TABLE 2

n	m	N/N_0
1	1	e^{-hD}
1	2	$e^{-hD}(1 + hD)$
2	1	$2e^{-hD} - e^{-2hD}$
1	3	$e^{-hD}(1 + hD + h^2D^2/2)$
3	1	$e^{-hD}(3 - 3e^{-hD} + e^{-2hD})$

The number of cellular entities involved cannot be deduced from the data available. The two simplest possibilities are: (1) Only one entity is involved, and its alteration requires the absorption, in or near it, of a minimal amount of energy which can be released either by one densely ionizing α particle or by two (possibly three) sparsely ionizing electrons. (2) Two (possibly three) entities are involved, all of which are altered by the same α track but only one of which can be altered by a single electron track. This is analogous to

certain chromosome aberrations (exchanges) whose dose-effect curves are one-hit with densely ionizing, and two-hit with sparsely ionizing, radiation (Catcheside, '47).

The clear-cut influence of linear energy transfer on the shape as well as the slope of the *Aspergillus* survival curve indicates that, of the successive events comprising the radiobiological action, the quantitatively decisive ones (Zirkle, '50) occur while each relevant ionization track still retains some of its original configuration and before the molecules initially activated by the high-energy particle (or the molecules or radicals derived from them) are scattered uniformly by diffusion. Thus, according to Lea's estimates ('47b) each decisive event probably occurs within a microsecond after passage of the relevant α particle or electron.

SUMMARY

After samples of air-dry spores of *Aspergillus terreus* had been given graded doses of polonium α particles and of 200 kv X rays, survival fractions were determined, the criterion of a surviving spore being the formation of a germ tube. When survival fraction was plotted against dose, the α-ray curves were exponential, but the X-ray curves were sigmoid and the experimental points could be fitted fairly well by "two-hit" theoretical curves. It is concluded that, in this particular radiobiological action, the number of entities which must be altered in each cell is quite small, and that, whereas all of them (one or more) are altered by one suitably located α track, two (possibly more) electron tracks are required to produce the same alteration(s). The results also indicate that the decisive events of the radiobiological action occur very early, i.e., before each relevant ionization track loses its original configuration by diffusion of the initially activated molecules or their chemical derivatives.

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MECHANISM OF LETHAL AND MUTAGENIC ACTION OF IONIZING RADIATION ON ASPERGILLUS TERREUS

I. RELATIONSHIP OF RELATIVE BIOLOGICAL EFFICIENCY TO ION DENSITY

GEORGE E. STAPLETON, ALEXANDER HOLLAENDER, AND FRANCES L. MARTIN

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

TWO FIGURES

As an integral part of an investigation concerning the mode of action of ionizing radiations on microorganisms, the comparative lethal and mutagenic action of heavy particles and X rays has been studied. Spores of the fungus, *Aspergillus terreus*, were used as test objects. Utilizing the technique developed by Hollaender et al. ('45), it was possible to obtain simultaneous information on inactivation and mutation induction on single, irradiated spore samples.

A survey of the literature indicates that information regarding inactivation of fungal spores by ionizing radiations is quite meager; moreover, the published data of several investigators are difficult to compare, chiefly because of basic differences in techniques utilized but also methods for estimation of the energy absorbed. It seemed advantageous to investigate the relationship of ion density to efficiency in producing not only inactivation but mutations as well, since the possible interaction of these two radiation effects has not been clarified. Every attempt was made to eliminate complicating factors, such as "clumping" of spores and presence of organic medium during irradiation.

MATERIAL AND METHODS

Spores from a subculture of the strain of *Aspergillus terreus* used by Hollaender et al. ('45) were employed as test organisms in all experiments. Normal spores from 14-day-old potato-dextrose-agar slant cultures were shaken into sterile tubes, from which small samples could be transferred to suitable sterile irradiation vessels. These vessels were small cylindrically shaped tubes, all of similar dimensions, 6 mm diameter $\times$ 20 mm long with 2 mm-thick walls. The material of which the tubes were fabricated depended to a great extent on the type of radiation used—lucite or polystyrene, for X-ray exposures; graphite, for neutron irradiation. All the exposure tubes were sterilized by placing them, open, beneath a G. E. ultraviolet germicidal lamp for approximately one hour previous to use.

After exposure to the radiation, the dry spores were shaken into sterile tubes containing isotonic saline solution to which a small amount of a wetting agent, sodium lauryl sulfonate, had been added in a concentration of about one part per million. To break up clumps, the suspension was shaken vigorously for 30 to 40 minutes in a special shaking device which agitated the suspensions from end to end of cork-stoppered test tubes at a rate of about 300 cycles per minute. At the end of the shaking period the suspensions were filtered through sterile absorbent-cotton filters, resuspended in saline, and refiltered, this process removing essentially all mycelial strands and spore clumps. Control samples were similarly prepared.

Since the use of dry spores necessitated an actual determination of the number of spores per unit volume of suspension, aliquots of the filtered suspensions were pipetted into a blood-cell-counting chamber and the spore count per milliliter determined by microscopic observation. Since the fractional counting error in such a procedure is inversely proportional to the square root of the number of items counted, the general practice was adopted of counting at least 2500 spores per suspension in order to limit the error to approximately 2%.

Once the spore concentration had been determined the suspension was appropriately diluted and plated on potato-dextrose-agar. Usually 4 plates were poured for each dilution and several dilutions plated to insure obtaining countable plates after incubation at 32°C. for 72 hours. Plates containing 30 to 300 colonies were considered countable.

The ratio of the product of the number of colonies per plate and the dilution factor to the actual spore count gave the surviving fraction, when corrected for survival among control lots. Samples of spores picked at random from experimental and control plates were transferred to small agar slants and incubated for 14 days at 32°C. Those colonies, macroscopically identifiable as different from normal control colonies, were classified as mutants; the types of mutants found included most of those described by Raper et al. ('45). All the mutants for which subsequent serial transfers were made retained the new morphological characteristics, which were distinct color changes in spores or mycelium, alterations in growth pattern, such as size, shape of colonies, or spore production. The ratio of mutant colonies to the total number isolated, corrected for spontaneous mutation rate (10^{-5} or less), determined the per cent of viable mutants.

RADIATIONS USED

X rays. The source of X rays used in this investigation was a G. E. Maximar 250 X-ray unit, equipped with an oil-cooled self-rectifying Coolidge tube. With the tube operating at 250 kvp and 15 ma, the X-ray dose measured 40 cm from the target was 300 r per minute with no added filtration, the inherent filtration being equivalent to 3 mm of aluminum. Dose measurements were made daily, prior to irradiation, at the same location occupied by the spore samples, with a Victoreen 100 r thimble chamber which had been recently calibrated by the manufacturer.

Fast neutrons. Uranium fission neutrons, which served as the source of fast neutrons in these experiments, were obtained

in the Oak Ridge reactor. The exposure system which was first described by Zirkle and Raper (to be published) consisted essentially of a sheet of normal uranium interposed between the biological material and a source of thermal neutrons, the reactor. Exposures were carried out in an aluminum cart which was inserted through the reactor shield immediately over the graphite lattice. The cart was so designed that all the thermal neutrons not used in fission were absorbed in a boron wrapper layer. Copious lead shielding was provided around the spore samples to insure essentially complete removal of those γ rays generated in the fission reactions, as well as those resulting from the boron-thermal neutron reactions. Because of the moderating effect of the layers of shielding the average energy of the bombarding neutrons was less than that expected for fission neutrons.

Dosimetry in regard to fast neutrons is probably the least developed in radiobiological research. No satisfactory system is available for estimating either fast neutron flux, where monoenergetic beams are not used, or the actual energy absorption in tissue.

Victoreen thimble chambers have been used by a number of investigators (Aebersold and Lawrence, '42; Giles, '43) to obtain approximate tissue dose measurements. This system involves a measurement of the ionization produced within the contained air by protons ejected from the thimble wall by fast neutrons. Since these heavy charged particles have relatively short ranges, they are absorbed by the graphite conducting lining of the thimble. Large differences have been reported between measurements made simultaneously with different chambers, the sensitivity of the chamber being determined to some extent by the thickness of the colloidal graphite lining.

Realizing that thimble-chamber dose measurements could involve some grave errors, this system was nevertheless adopted, with the expectation that some correlation of the n unit (Evans, '47) as measured, and biological efficiency might result from this study. A 100 r thimble chamber previously

used in this laboratory for neutron dosimetry was used in these experiments.

The dose of neutrons, measured in the position occupied by the spores was 2.7 n per minute. The gamma contamination in the exposure system minus the uranium was 0.27 r/min. at the pile operating level used in these exposures.

Thermal neutrons. Since thermal neutrons are by definition extremely low-energy uncharged particles (0.025 ev in a graphite-moderated reactor) they are unable to produce any ionization of themselves, but more interestingly, exert their biological effect as a result of being captured by nuclei of atoms present in the matter bombarded. The compound nucleus, resulting from capture of one of these neutrons, has excess energy which must be eliminated by emission of a particle (neutron, proton, or α-particle) or an energetic photon. As Conger and Giles ('50) have indicated, the compond nuclei of most importance are those which eject a heavy charged particle and are thrown back some short distance, producing intense local ionization.

In biological materials the most important reactions are

$$(1) \qquad H^1 (n,\gamma) H^2$$

because of the abundance of hydrogen in normal tissue,

$$(2) \qquad N^{14} (n,p) C^{14}$$

chiefly because of the energetic proton emitted, rather than abundance of the element, or capture cross section, and

$$(3) \qquad B^{10} (n,\alpha) Li^7$$

because of the very high capture cross section and the emission of a heavy charged particle.

The other atoms present contribute relatively little to the total ionization produced by thermal neutron bombardment and can be neglected.

Table 1 shows some of the important characteristics of the reactions which lead to the biological effects produced when spores of this fungus are bombarded with thermal neutrons.

The dose of radiations from the several reactions can be estimated by use of equation 1.

$$\text{Rep/min} = N \times F \times \sigma_c \times E \times A \times \text{ergs/ev} \times \frac{1}{83} \qquad (1)$$

Where N = the number of atoms of the element present/cm^3
F = thermal neutron flux in neutrons/cm^2/minute
σ_c = capture cross section for thermal neutrons
E = energy of the emitted radiation in electron volts
A = fraction of energy absorbed
Ergs/electron volt = 1.6×10^{-12}
and 1 rep is equal to 83 ergs of energy absorbed per gram of tissue.

It is clearly seen from table 1 that the slow-neutron exposures carried out with this material consist essentially of α radiation, approximately 82% of the total energy absorbed being due to the boron capture reaction and 96% of the total energy absorption resulting from heavy particle reactions. Conger and Giles ('50) mentioned previously that one of us (G.E.S.) had attempted a measurement of γ-ray contamination inherent in the exposure system. These measurements indicate an unexpectedly high contamination, approximately 3–6 r/minute, which increases the total dose to about 24 rep/minute.

The thermal neutron treatment chamber located in the Oak Ridge reactor has been described thoroughly by Conger and Giles ('50). In the same publication the methods of determination of flux have been discussed and, since the same general procedure was used in these exposures, no further discussion will be given here. The several radiations used are summarized in table 2.

RESULTS

Inactivation. The data regarding the comparative lethal effects of α-particles, fission neutrons, and X rays are plotted graphically in figure 1. The individual values plotted are averages of several experiments each, the standard deviations being about 5%. The logarithm of the fraction of spores surviving is plotted as a function of dose of the radiations. The units of dose for the various radiations are expressed in roent-

TABLE 1

Thermal neutron reactions in normal spore material

ELEMENT	ATOMIC WEIGHT	PER CENT WET WEIGHT OF ELEMENT	GM ELEMENT PER GM TISSUE	ATOMS PER $CM^3 \times 10^{24}$	CAPTURE CROSS SECTION $\times 10^{-24}$ CM^2	RADIATION TYPE	EMITTED ENERGY (EV)	FRACTION OF ENERGY ABSORBED	REP PER MINUTE	PER CENT OF TOTAL ENERGY ABSORBED
Hydrogen	1.008	10.0	0.10	6.24×10^{-2}	0.32	Gamma	2.24×10^{6}	0.01	0.64	3.6
Nitrogen	14.008	3.1 [a]	0.03	1.28×10^{-3}	1.75	Proton	0.63×10^{6}	1.00	2.60	14.6
Boron	10.820	1.05×10^{-2} [b]	0.000105	5.86×10^{-6}	715.0	Alpha	2.4×10^{6}	1.00	14.41	81.7
									Total 17.65 rep/minute.	

[a] Total nitrogen determination.
[b] From spectroscopic analysis of spores.

TABLE 2

Physical characteristics of the radiations

RADIATION	SOURCE	ENERGY	INTENSITY
X rays	G. E. Maximar 250 unit inherent filtration — 3 mm Al 250 kv — 15 ma half-value layer = 0.4 mm Cu	250 kvp	300 r/minute
Protons	Fission neutrons (O. R. reactor)	~ 1 mev	2.7 n/minute
	γ contamination		0.27 r/minute
α-Particles	Thermal neutrons (O. R. reactor)		
	(1) B^{10} (n,α) Li^{7} [a]	2.4 mev [a]	14.4 rep/minute
	(2) Plus protons N^{14} (n,p) C^{14}	0.63 mev	2.6 rep/minute
	(3) Plus γ rays	2.24 mev	0.64 rep/minute
	(4) Plus γ rays inherent in chamber	?	3.0–6.0 rep/minute

[a] The lithium recoil and particle share this kinetic energy in an inverse ratio to their relative masses.

gens for X rays, roentgen equivalents for α-particles, and in n units for neutrons.

Figure 1 clearly indicates that survival is a different function of dose for sparsely ionizing radiation from either of the

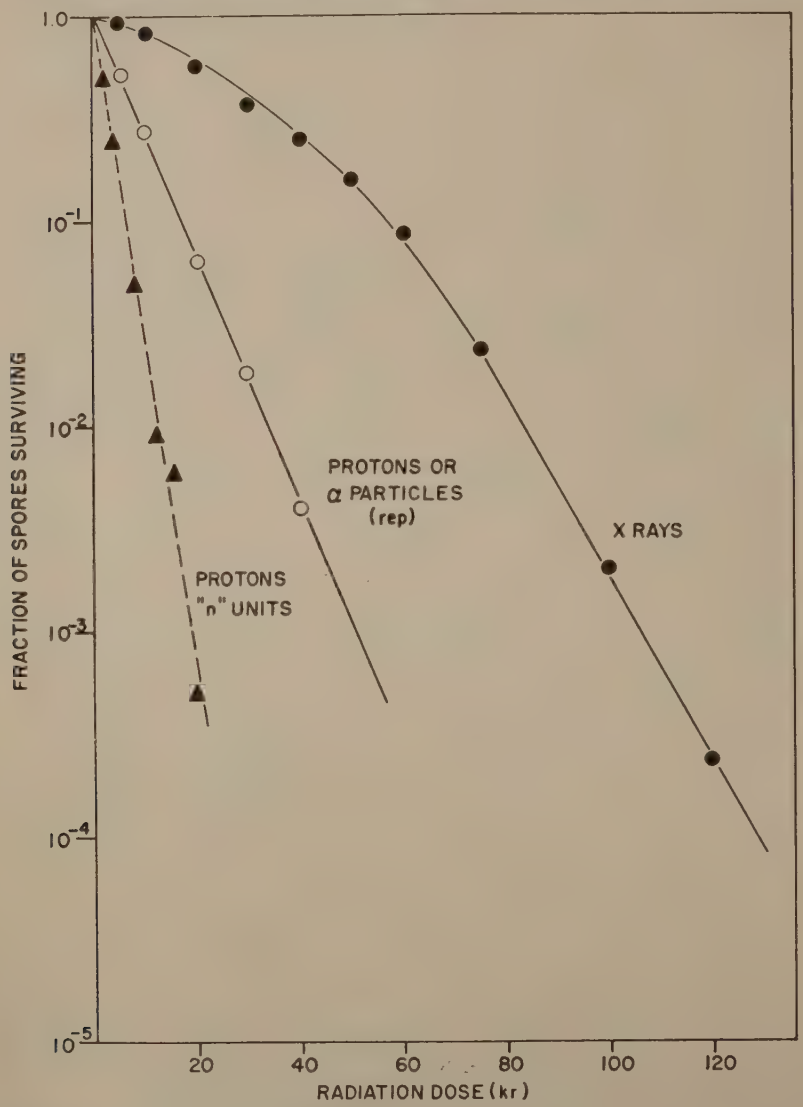

Fig. 1 Comparative lethal effects of X rays, protons, and α-particles on *Aspergillus* spores.

heavy ionizing particles. Within experimental error the curves for both α-particles and protons are exponential, whereas a definite sigmoid type of curve is obtained for X rays, with a "hit number" of approximately 3. These data are consistent with the hypothesis that several electrons are required to produce the same quantitative effect as a single α-particle or proton.

It is of interest that a comparison of the curves for the heavy particles indicates that 1 n of fast neutrons is equal to approximately 2.5 rep of α-particles, which is the same frequently mentioned factor for conversion of the n unit as measured with the Victoreen thimble chamber to tissue energy absorption in roentgen equivalents. This is perhaps fortuitous, since it assumes that α-particles have no increased efficiency over protons, and that the lithium recoil is no more biologically efficient than the coincident α-particle.

MUTATION PRODUCTION

Figure 2 is a plot of per cent observed viable mutants as a function of radiation dose for α-particles and X rays, the energy absorption expressed in roentgens or roentgen equivalents. The relative efficiencies of the two types of radiation for production of mutants is apparently a complete reversal of that found for the lethal effect.

The curves relating mutation frequency to energy absorbed are linear over a rather wide range. Since the α irradiation included a large γ contamination, about 25% of the total dose, it seemed desirable to correct the α-particle mutation curve for this contamination, assuming γ rays to be as efficient as X rays in producing mutations. The results indicate that X rays are approximately twice as efficient per unit dose as the heavily ionizing α-particles in producing morphological mutants in this strain of fungus.

There is some suggestion that, at X-ray dose levels above 60 kr (and up to 120 kr), a second mutation mechanisms is involved, the yield of mutants increasing more rapidly than the

first power of dose. This will be discussed in more detail in a later section.

DISCUSSION

It must be realized at the beginning that neither cytological examination nor genetic testing is possible with this spore material. Nevertheless, it seems somewhat appropriate to cite as a foundation for speculation some radiobiological phe-

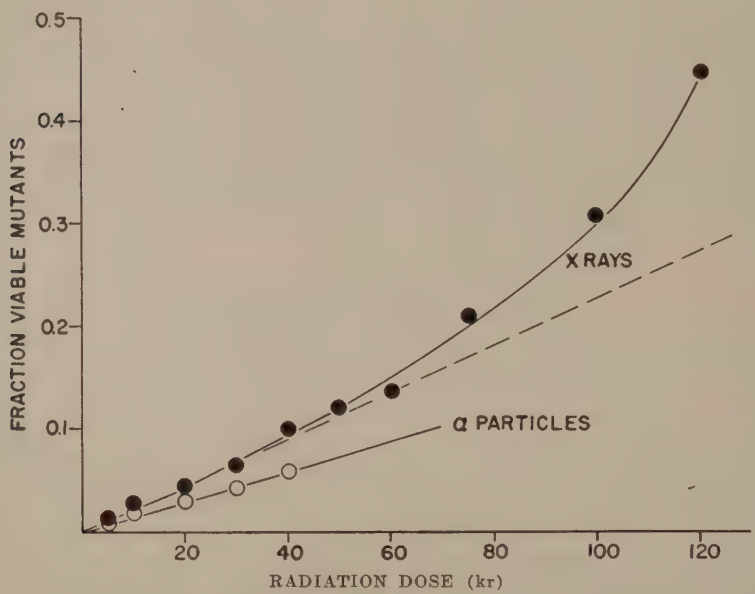

Fig. 2 Viable mutation rate with X rays and α-particles.

nomena which have been shown to have similar relationships in terms of energy absorption from ionizing radiation to those found for inactivation and mutation in this investigation.

First of all, we have shown that the heavy ionizing particles, which are more efficient lethal agents than electrons, are less effective mutagenic agents. Moreover, the rate of mutation with X rays or α-particles has a linear relationship with dose over a wide range, indicating that a process akin to gene mutation probably can account for many of the morphological mu-

tants recovered. The lesser yield per unit dose with heavy particles has already been shown by several investigators, and reviewed by Lea ('47).

The apparent change in the order of the mutation curve in the higher dose range has already been mentioned, and resembles to some extent the relationship obtained for two-hit chromosomal aberrations studied by Sax ('41) and others. Swanson ('48) has suggested, on the basis of his experiments with modifying agents, that chromosomal aberrations play a role in mutation production in this species of *Aspergillus*. Minute deletions could result in production of mutations, some of which would be expected to yield a two-hit relationship with sparsely ionizing electrons.

It is of interest that an increase in the X-ray mutation rate is apparently coincident with an increase in inactivation rate, above 60 kr, which perhaps indicates a similar fundamental mechanism of lethal and mutagenic action at the higher dose levels. There are several alternative explanations for this phenomenon, all of which may be resolved into one with more extensive investigation. The inactivation curve can easily be fitted with a sigmoid curve or a pair of exponential curves with slopes different by a factor of about 4. In any case, the X-ray inactivation curve becomes exponential in the dose range above 60 kr, within the error of experimentation. The nature of the curve in either case indicates, in part, a cumulative type of action, which could be interpreted on the basis of the direct effect, to mean that several electrons are required to produce the effect. Lea ('47) and others explain an effect similarly related to dose (chromosomal aberrations of the two-hit type) by assuming that only electron tails can produce the breakage, and the probability being less than unity. An interesting alternative explanation for the survival curve obtained for X rays is that the inactivation of these spores is accomplished by both direct and indirect action, the latter resulting from some toxic substance produced by the radiation within the spore, a threshold concentration being required to inactivate.

Consistent with the idea of chromosomal effects being partly responsible for the lethal effect are the findings of Zirkle ('49) that much higher doses of both α-particles and X rays are required to prevent germination of spores of *A. terreus* than are required to prevent colony formation. Comparison of Zirkle's data with ours indicates that, at any dose, many more spores germinate than can form visible colonies, suggesting that many spores die in the process of division. Koller ('43) has presented evidence that viability of *Tradescantia* microspores can be correlated with loss of acentric fragments.

Sansome et al. ('45) have shown an intensity effect for mutation production in *Neurospora,* which seems to indicate that some process other than gene mutation plays a role in the mutagenic actions of X rays in that fungus, since no intensity effect has yet been established for gene mutation.

In our experiments no particular type of mutant was found, the frequency of which was differentially increased at higher dose levels, the trend being a general increase in all types of morphological mutants.

The data presented for X-ray effects seem to indicate that an indirect action may account for some of the lethal and mutagenic action. If an indirect action is responsible in part for the lethal effect, then the increased efficiency of the protons and α-particles may be explainable on the basis that sufficient concentration of the toxic product occurs locally by the passage of a single densely ionizing particle, whereas several electron tracks are required to produce the required concentration; hence the cumulative action of X rays. Moreover, the lesser yield of mutations per unit dose with the heavy particles might be expected in the light of such a mechanism, if the mutations are produced by a direct action, and this process must compete with the indirect process, the recovery of viable mutants being the result of the over-all reaction.

An apparent change of slope in the mutation curve was observed at high X-ray dose levels, which might be interpreted as indicating a cumulative type of action for mutation production; an indirect effect possibly occurs here as well as in

the inactivation process. Such indirect effects are subject to test by means other than direct observation and will be treated in Part II of this paper.

SUMMARY

1. Densely ionizing α-particles and protons have been found to be more efficient than X rays in inactivating spores of *A. terreus*.

2. A change of shape of the survival curves occurs with increasing ion density.

3. A reversal of the efficiency versus ion-density relationship was demonstrated for production of viable mutations.

4. The type of survival curves obtained can be explained on the basis of the "multiple hit" target hypothesis, or on the basis of an intracellular indirect effect, a threshold concentration of a radiation-produced toxic substance being required to inactivate the spores.

5. The mutation curve of X rays suggests that perhaps two mechanisms may be involved in production of viable mutations in this strain of *A. terreus*.

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MECHANISM OF LETHAL AND MUTAGENIC ACTION OF IONIZING RADIATIONS ON ASPERGILLUS TERREUS

II. USE OF MODIFYING AGENTS AND CONDITIONS

G. E. STAPLETON AND ALEXANDER HOLLAENDER
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

THREE FIGURES

In Part I of this study data were given concerning the relationship of ion density to efficiency of several radiations in production of the lethal and mutagenic response in spores of *Aspergillus*. Of utmost interest is the relationship between energy absorption of X rays for both inactivation and mutation. It appears from the data presented that chromosomal aberration, or an intracellular indirect effect, plays a significant role in both killing and mutagenesis.

Since neither genetic testing nor cytological examination is possible with these relatively small cells, other approaches have been used in an effort to gain further insight into the mechanism of biological action of X rays. One approach which has been fruitful, where direct observation is not possible, is through the use of agents or conditions which in themselves produce no detectable deleterious effect, but which do alter the radiosensitivity of cells. Of these agents or conditions, several have been demonstrated conclusively to alter the sensitivity of cells to X radiation. Regarding physical agents or conditions, Swanson et al. ('48), Kaufmann et al. ('46), and Hollaender et al. ('47) have shown that infrared pretreatment alters the sensitivity of a variety of organisms to X rays as well as to ultraviolet. For some radiation-produced biological effects, the temperature of the biological

material during exposure to X rays determines to some degree the sensitivity of the material. Baker ('49) has shown that, at various temperatures, the radiation mutability of one strain of *Drosophila* is altered, the trend being toward greater sensitivity at low temperature. Glücksmann and Spear ('39) demonstrated that cell degeneration in the tadpole eye was reduced when irradiation took place at temperatures low enough to reduce metabolism, and hence mitotic activity. Normal degeneration took place, however, when tadpoles were again brought into room temperature and mitosis resumed. Numerous chemical protective agents have been used. Barron ('49) and Dale ('47) have shown that organic compounds, especially those containing sulfhydryl groups, are active in protecting isolated enzymes against X-ray inactivation. Moreover, Barron has demonstrated reversal of X-ray inhibition of sulfhydryl-requiring enzymes by posttreatment with glutathione. Lea ('47) was able to protect tobacco mosaic virus almost completely against the indirect effect of X rays by addition of gelatin or other proteinaceous material. A striking decrease in X-ray sensitivity of the living cells by lowering of oxygen tension has been observed in many investigations; Mottram ('47), in tumors; Anderson ('41), on yeast; Hayden and Smith ('49), on barley seeds; Thoday and Read ('47), on bean roots; Baker ('50), on mutations in *Drosophila;* Giles and Riley ('49), on chromosomal aberrations in *Tradescantia;* Gaulden ('50), on mitotic inhibition in grasshopper neuroblasts; and Hollaender and Stapleton ('51), on inactivation of bacteria. Change in sensitivity, dependent on water content has been demonstrated by Stadler ('28) and Henshaw ('35) on seeds and by Lea ('47) on plant viruses.

The spores of fungi are especially well adapted to an investigation of the relationship of water content to radiosensitivity since, similarly to seeds, the relative water content can be varied over a wide range without altering the viability of the cell. Since it has been suggested (Lea, '47) that radiodecomposition of water by X rays could account for some lethal effects as well as some mutagenic effects, it was decided

to study not only the relationship of sensitivity to relative water content but also the oxygen tension effect on this system.

MATERIAL AND METHODS

Since it was of interest here to relate water content to radiosensitivity, three systems were set up in this study, essentially as follows:

Low water content. The desiccated spore water content was determined by drying over drierite *(in vacuo)* to a constant weight. These weights were then compared with totally dried material (oven dried).

Medium water content. The relative water content of normal spores was determined by drying to a constant weight at 110°C. in an electric oven. Several 30–100 mg samples gave an average of 43% weight loss by this treatment.

High water content. The water content of suspended spores was not measured. The average values for determinations on water content of mycelial masses containing spores was taken from Porter ('48), which is considered by many investigators to be about the water content of wet tissue.

Since the oxygen-tension effect on radiosensitivity was studied as part of this work, it was necessary to design a system which would allow spore samples to be evacuated before introduction of the appropriate gas. Small (2 ml), thin-walled volumetric flasks with ground stoppered tops were fitted with ground jointed microstopcocks which permitted evacuation of the system and subsequent introduction of an inert gas (nitrogen) around the spores. The tubes for suspensions were essentially similar to those used for dry spores, except that provision was made for bubbling the gas through the suspensions. These small irradiation vessels were inserted into a lucite holder which accommodated eight such tubes, holding them in a predetermined uniform field under the X-ray tube. The holder was designed to hold a sufficient quantity of an ice-water mixture to insure a constant temperature of biological material during X-ray exposure. All

spore samples were allowed to cool in the tubes at ice-bath temperature for approximately 30 minutes prior to irradiation. Figure 1 is a diagram of the irradiation vessel and holder.

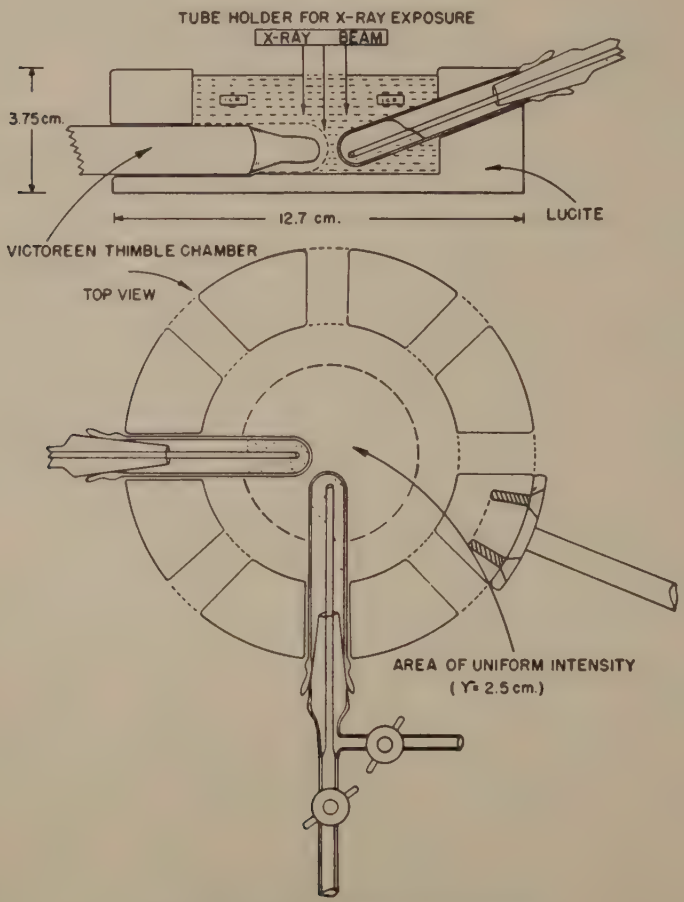

Fig. 1 Irradiation vessel and holder.

The general procedure outlined in Part I of this study was used for normal and desiccated spores following irradiation. In those experiments involving use of suspended spores, suspensions were prepared prior to irradiation.

In preliminary studies it was found that a good estimate of the mutation frequency could be obtained by merely holding plates, on which there was no crowding, for 10 days at room temperature. A second count of those colonies different in morphological characteristics from the normal, as compared with the total count, gave the fraction mutated. In several experiments the frequency obtained by this type of estimation was checked against the frequency obtained by isolating individual colonies and was found to compare favorably, to within 10%. This technique was used in this portion of the investigation.

RADIATION USED

This investigation was restricted to X-ray inactivation and mutation. The source was a G. E. Maximar 250 unit equipped with an oil-cooled, self-rectifying Coolidge tube (inherent filtration 3 mm of Al). The dose in the position occupied by the spore samples was measured daily prior to irradiation. With no added filtration, the dose as measured at 40 cm from the target with a 250 r Victoreen thimble chamber recently calibrated by the manufacturer, was 300 r per minute.

RESULTS

Relation of water content to radiosensitivity. Figure 2 indicates quite clearly that the sensitivity of spores is dependent to a great extent on the relative water content during the time of exposure. The sensitivity, based on the dose necessary to produce the same quantitative lethal response is shown in table 1.

These data indicate that sensitivity of spores to X radiation is dependent on the relative water content of the spores. This finding is consistent with the earlier ones of Stadler ('28), Henshaw ('35), and Lallemand ('32) that much larger doses of radiation are required to inactivate dried seeds than moist ones. Similarly Lea ('47) was able to demonstrate that, for purified tobacco mosaic virus, the inactivation dose for dilute

suspensions was several times less than that for dried preparations. The explanation offered was that in aqueous suspension the indirect (a mediated effect through energy absorp-

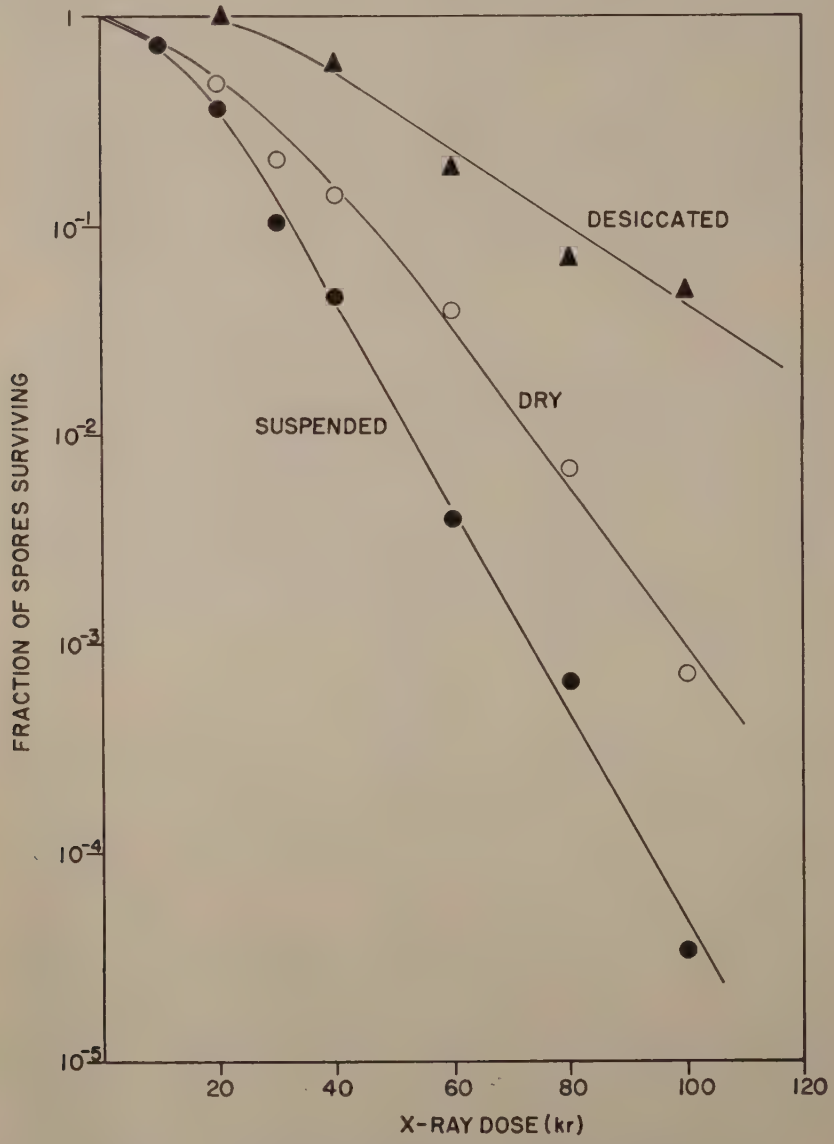

Fig. 2 Relation of water content to X-ray sensitivity.

tion by water molecules) and direct effects are additive in producing virus inactivation.

When spores are irradiated in oxygen-free suspensions the indirect effect is decreased strikingly. This is evidenced by comparison of dry spores in oxygen atmosphere with oxygen-free, nitrogen-saturated suspensions in table 2. In the same table, it will be noted that removal of oxygen from dry spores prior to irradiation, by evacuation and subsequent replacement with an atmosphere of nitrogen, reduced their sensitivity but not as strikingly as the removal of oxygen from suspensions of spores. This further reduction of sensitivity of dry spores which has no aqueous surrounding, sug-

TABLE 1

Relationship of radiosensitivity to relative water content

	DESICCATED	DRY	SUSPENDED
Per cent water by weight	~ 25	~ 42	~ 80
Ratio of water contents	1	1.8	3.2
Dose in kr to kill 99%	126	74	52
Ratio of sensitivities	1	1.7	2.4

gests the importance of intracellular water in X-ray inactivation of spores. This will be discussed in more detail in a later section.

Mutation. Even though the method used in this investigation for determining the frequency of mutation induction was not the same as that used previously, the X-ray yield per unit dose is not significantly different in that system which is common to both, viz., X irradiation of dry spores.

Figure 3 shows the relative rates of production of mutants under the different conditions studied. It is interesting that decreasing the water content of the spores or elimination of oxygen from suspended spores tends to reduce the over-all mutation frequency. The mutation reduction phenomenon is especially striking at high dose levels. It is apparent from these data that either an over-all quantitative reduction in

the yield of mutations per unit dose is obtained by removing water from the spores or removing oxygen from suspended spores, or else there is a specific reduction of those "second-order mutants" obtained normally at the higher dose levels. These data do not allow us to determine which of these effects occurs. In any case there appears to be a mechanism for X-ray mutation which has similar characteristics to the process involved in X-ray inactivation. Our methods did not permit resolution of any particular type of mutant for which the frequency had been altered differentially by the aforementioned conditions.

TABLE 2

Oxygen tension effect on radiosensitivity of dry and suspended spores

DOSE (r)	FRACTION SURVIVING			
	Dry spores		Suspended spores	
	Oxygen	Nitrogen	Oxygen	Nitrogen
20,000	0.489	0.726	0.365	0.713
30,000	0.205	0.402	0.103	0.416
40,000	0.137	0.417	0.0485	0.243
60,000	0.0402	0.135	0.0041	0.043
80,000	0.0069	0.0508	0.00066	0.0167
100,000	0.00070	.....	0.000034	0.00176
120,000	.....	0.002	0.0000024	0.00060

DISCUSSION

It has been shown by previous investigations that striking changes in X-ray sensitivity, as it concerns lethal effects, physiological effects, as well as effects on the genetic makeup of the cell, can be obtained by simple alteration of the physical conditions at the time of irradiation, or by pretreatment of the biological material with various physical or chemical agents.

Two simple changes in physical or chemical conditions, at the time of exposure of *Aspergillus* spores to X rays, have resulted in radical modification of sensitivity in regard to inactivation as well as mutation in this investigation. The

data presented indicate that the dose required to inactivate the same fraction of spores is inversely related to their relative water content. One might suggest, on the basis of the "target hypothesis," that swelling or shrinking of the spores by suspension or desiccation, respectively, could alter the sensitivity of these cells. However, removal of oxygen from the cells, which should not alter the target, nevertheless, similarly reduces the sensitivity of the cell. The most logical

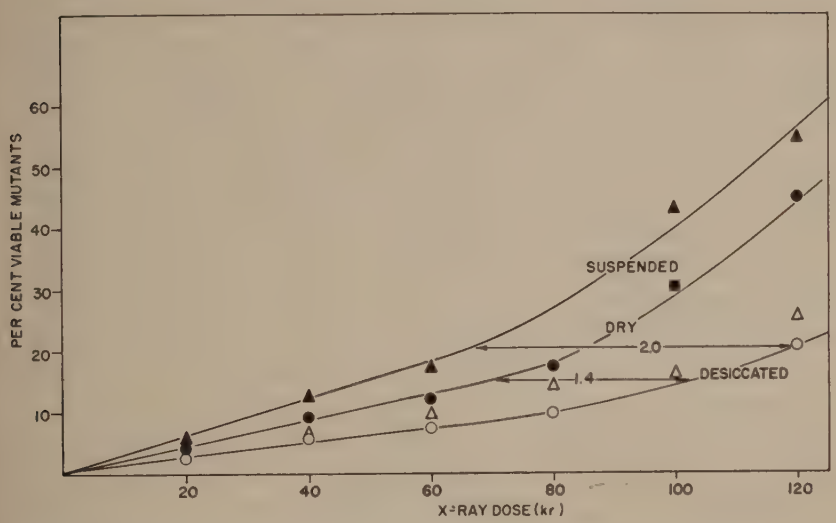

Fig. 3 Frequency of morphological mutants among surviving spores of *Aspergillus terreus.*

▲ — Suspended (oxygen-saturated buffer).
● — Dry — in oxygen atmosphere.
○ — Desiccated.
△ — Suspended (nitrogen-saturated buffer).

explanation for both the relationship of relative water content and oxygen tension to X-ray sensitivity is found in the suggestions of Weiss ('47), Lea ('47), and others, that radiodecomposition products of water could be biologically effective toxic agents. The production of some oxidizing intermediates in the decomposition of water under the action of ionizing radiations, such as O_2H and hydrogen peroxide, would, ac-

cording to Weiss' scheme, be produced in relatively high yields only in water containing dissolved oxygen.

It is interesting in this regard that replacement of oxygen by nitrogen in dry spores (40–45% of water by weight) also resulted in a reduction of sensitivity. In this system no extracellular water exists, hence there is no possibility of an indirect effect transmitted through the medium. There is a possibility that reactions occur in the intracellular material similar to those that occur in the extracellular water in spore suspensions. These reactions may result in production of active radicals as well as peroxides. The yield might be low because of the presence of dissolved organic substances; however, the low yield might be compensated for to some extent by the ideal position of these products for reaction with important biological centers. Gray ('47) suggested that chromosomes might be damaged by active radicals produced in the water in and around them during irradiation.

It is interesting that, as suggested in Part I, part of the mechanisms for mutation and inactivation with X rays have some similar characteristics. In this portion of the work we have shown that both are controlled to a great extent by relative water content and oxygen tension during irradiation. It is difficult to reconcile these findings with the hypothesis that direct hits on important biological entities can explain completely either inactivation or mutation under the conditions used in this investigation. No clear-cut method of assessing the relative importance of either the direct or indirect effects seems obvious from this study, since, from all indication, the two effects have different dose relationships, the indirect effect appearing always to have a threshold. This is evident from inactivation data. Assuming that there are two mutagenic mechanisms having different dose-frequency relationships, the higher order mutation curve obviously has a threshold dose. By none of the treatments used have mutations been eliminated, both desiccation and removal of oxygen resulting in a more nearly linear relationship of dose to muta-

tion frequency. One might assume that these linear curves represent the mutations produced by direct hits on genes. Similarly the inactivation curve for desiccated spores might be a reflection of the killing action produced by direct hits.

The relative importance of indirect effects in production of the lethal or mutagenic effect by heavy particles remains to be investigated. According to the scheme suggested by Weiss ('47) and others reduction of the oxygen tension during irradiation with α-particles should not alter the production of hydrogen peroxide or other oxidizing decomposition products resulting from ionization of water.

It is not clear whether reduction of the water content, as performed in these experiments, would alter the production of such toxic agents, since bound water may be of some importance in production of free radicals and other reactive chemical species.

The results reported in this investigation certainly emphasize the importance of an intracellular indirect effect in the production of at least a part of the inactivation as well as the mutation in the spores of this fungus.

SUMMARY

1. A direct relationship has been found between X-ray sensitivity of spores of *Aspergillus terreus* and their relative water content.

2. A reduction of inactivation as well as mutation was obtained also by lowering of oxygen tension in and around spores during X irradiation.

3. It would appear from this investigation that there are two mechanisms of production of mutations by X rays in this material; one affected strikingly by removal of water or oxygen prior to irradiation, the other seemingly unaffected.

4. The findings of this investigation are consistent with the hypothesis that indirect effects play a major role in inactivation as well as mutation.

5. The importance of reactive decomposition products of water, produced intracellularly by X irradiation is indicated.

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EXPERIMENTS ON PHOTOREACTIVATION WITH BACTERIA AND OTHER MICROORGANISMS

ALBERT KELNER[1]

The Biological Laboratory, Cold Spring Harbor, Long Island, New York

In a population of microbial cells irradiated with ultraviolet light (2537 Å) a large portion of the cells are inactivated; that is, they become nonviable, and are unable to form a colony (the chief experimental criterion for inactivation), or undergo more than a few divisions, if any, before dying. If such inactivated cells are illuminated with visible light, a large portion of these cells recover their viability, or are reactivated, and are able to divide apparently normally and form a colony. The process of light-induced recovery from ultraviolet irradiation injury is called photoreactivation.

Since the discovery of photoreactivation (Kelner, '49) we have been conducting exploratory studies of various fundamental aspects of the phenomenon, chiefly with the bacterium *Escherichia coli.* An intensive study on the genetic aspects of photoreactivation in *E. coli* is now in progress, and hence will not be discussed in this lecture. We will, however, discuss several of the general characteristics of photoreactivation among microorganisms.

Action of visible light. The question exists as to whether visible light acts upon compounds within the cell or on toxic materials in the external menstruum. That the reactivating visible light affects a substance or substances within the cells is shown most conclusively by an experiment in which the entire cycle of ultraviolet inactivation and visible light reactivation was carried out on dry actinomycete spores. Since the external menstruum in this case was air, all the reactions must have taken place on or within the cell itself.

[1] Present address: Brandeis University, Waltham, Mass.

Generality of photoreactivation. This phenomenon has been found in all microorganisms tested by us, including *Streptomyces griseus, Escherichia coli B/r, Saccharomyces cerevisiae,* and *Penicillium notatum.* Our discovery has been beautifully confirmed by Dulbecco for bacteriophage, and by other workers for *Neurospora.* There can be no doubt, then, that the reaction is general for most microorganisms.

Effective wave lengths. Preliminary experiments with the use of Wratten filters for photoreactivation of *E. coli* have shown that the active wave lengths lie below 5100 Å. Most experiments have been carried out in a room illuminated with only enough yellow light to enable the experimenter to work with ease, yet with no danger of unwanted photoreactivation.

Kinetics of photoreactivation. Photoreactivation is proportional to light intensity and duration, within limits, and proceeds more rapidly with a rise in temperature. Photoreactivation does occur when cells are illuminated at 5°C. The Q_{10} for actinomycetes is about 3, for the range between 20° and 40°C. Very prolonged illumination of ultraviolet-irradiated *E. coli* cells, especially in the cold, has a lethal effect on such cells. This suggests the possible presence of a secondary lethal effect of visible light. The active wave lengths for the latter effect may not necessarily be the same as for photoreactivation.

Recovery in the dark. Slight recovery has usually been observed in ultraviolet-irradiated *E. coli* cells kept at 37°C. for 45 minutes. The increase in survival rate has been about two- to threefold as compared to a 5,000- to 10,000-fold increase for controls in the light.

Reduction of the lag phase. Photoreactivated cells are not only viable, but their lag phase is shorter than that of the initial survivors of ultraviolet-irradiated suspensions. The lag phase of the photoreactivated cells is, however, equal to or longer than that of normal nonirradiated cells. The shortened lag phase was most noticeable in *P. notatum.*

Loss of recoverability. When ultraviolet-irradiated *E. coli* cells are put into a favorable medium at 37°C., in the dark,

they lose in three hours their ability to recover upon subsequent illumination. This loss in recoverability suggests that a study of the changes that go on during this three-hour period may offer information as to the cell compounds concerned in photoreactivation.

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REACTIVATION OF ULTRAVIOLET-IRRADIATED BACTERIOPHAGE BY MULTIPLE INFECTION

S. E. LURIA[1]

Department of Bacteriology, Indiana University, Bloomington

For a number of years, we have employed radiation in our laboratory as a microsurgical tool to analyze properties and growth of bacteriophages. In general, radiation appears to suppress reproductive ability of phage at a faster rate than other phage properties, a fact utilized in dissociating the latter properties from the former one. Also, radiation was used to follow the phage in the course of its intracellular growth (Luria and Latarjet, '47). In all this work, loss of reproductive ability ("inactivation") was considered as an irreversible change affecting the phage particle as a whole. What is observed, however, is actually the loss of ability to initiate production of active phage *under given conditions.* Results obtained in our laboratory in the past two years indicate that these conditions have to be defined more precisely before the term "inactivation" can be used in a meaningful sense. Moreover, it may be possible to localize the changes involved in inactivation more precisely than in the virus particle as a whole. The new finding is that active virus can be produced from particles that would ordinarily be considerd inactive in a number of clearly definable situations.

The first case to be discussed in this paper is that of multiplicity reactivation (Luria, '47); photoreactivation will be discussed by Dulbecco. Ultraviolet-irradiated coli phages (T2, T4, T5, T6) are still adsorbed by sensitive bacteria at approximately normal rate (irradiated phage T1 is less well adsorbed). The active titer, measured by plaque count after

[1] Present address: Department of Bacteriology, University of Illinois, Urbana, Illinois.

mixing with a very large excess of bacteria (single infection) diminishes logarithmically with the dose. The irradiated particles can then be divided into two classes: (a) those that give rise to production of active phage following single infection (active particles); (b) those that upon single infection do not give rise to active phage (inactive particles). It was found that, for all the phages listed, active phage may be produced by bacteria infected by more than one of the "inactive" particles. Active phage of a given type, for example T2, can also be produced if one "inactive" particle is taken up by a bacterium along with an active particle of a related phage (T4 or T6), not of an unrelated phage.

The phages T2, T4, and T6 are those for which recombination of genetic characters upon mixed infection have been demonstrated (Delbrück and Bailey, '46; Hershey and Rotman, '48, '49). These recombinations can most easily be interpreted in terms of recombination of discrete material determinants. An appealing working hypothesis to explain reactivation was, then, that active phage was produced from inactive particles by genetic recombination. The "inactive" particles would be those in which radiation had inactivated certain determinants. This would be those replaceable by homologous active determinants ("units") supplied by other particles infecting the same bacterium.

A number of simplifying assumptions permitted quantitation of this hypothesis in such a way as to yield predictions on how the probability of active phage formation in a bacterium depends on the number of infecting inactive particles and on the dose of ultraviolet light they had received ("simple theory"). The assumptions consisted mainly in postulating (a) a fixed number of damageable and replaceable units per phage particle, (b) identification of the average number of damaged units per particle with the natural logarithm of the survival ratio (number of "hits"), and (c) a minimum requirement for active phage production of at least one undamaged copy of each unit among the infecting particles

taken as a group. An additional nonessential assumption is that of equal ultraviolet sensitivity for all units.

These assumptions led to an expression of the probability of active phage production that could be tabulated numerically and plotted in curves. A number of such curves (Luria and Dulbecco, '49) are shown and compared with the experimental values of the frequency of active phage production. The general trends of theoretical and experimental values are similar. There is, however, a systematic deviation, in that the experimental frequency lags behind the theoretical probability as the number of infecting particles increases. This might be interpreted as suggesting a low degree of cooperation among several particles in the same bacterium. The striking fact is that the frequency of reactivation per multiple infected bacterium still remains near unity with particles that have received a fairly large number of "hits." If the working hypothesis of localized damage and recombination of undamaged units is correct, then the high frequency of reactivation can only be interpreted as the result of an extremely efficient mechanism of recombination, which can bring together all undamaged units.

As one such mechanism, I suggested ('47) independent replication of the active units followed by reassembly of active particles. This hypothesis agrees with the accumulating evidence for an early period after phage infection, in which active particles are either not present or at least not recoverable from the infected bacterium (Doermann, '48). No evidence is available for replication of parts of inactive particles in bacteria, in which reactivation does not take place. That deep changes take place in these bacteria, however, is shown by interference phenomena, suppression of bacterial growth, and cytological changes (Luria, Human, and Robinow, unpublished).

It may be added that the frequency of reactivation as a function of the dose of radiation is characteristic for each phage. From the "simple theory" we can calculate a minimum number of discrete, damageable, and transferable units

per particle (defined according to our assumption). These numbers vary from 25 to 30 for T2 and T6 to around 15 for T4, possibly 10 for T5 and less than 4 or 5 for T1. It must be understood clearly that these values are meaningful only if the hypotheses of the simple theory are justified, which remains to be confirmed.

Since the whole theory hinges on the concept of damage in discrete units of genetic material, one attempt at confirmation consisted of experiments using genetically marked strains of phage T2 (Dulbecco and Luria, to be published). Bacteria were infected with one particle of an active strain and one particle of an irradiated strain, the two strains differing in a pair of alternative characters. Each bacterium thus infected should liberate a mixture of the two infecting types. Failure to liberate the type corresponding to the irradiated parent could indicate inactivation and loss of the locus corresponding to the marker character. The proportion of bacteria liberating a mixture of the two types was determined and a loss was found for several loci. The frequency with which a locus was recovered decreased more or less logarithmically with increasing doses of radiation.

Some questions on the interpretation of these results were raised, however, by experiments in which the yields from individual bacteria infected with one active and one irradiated particle differing in one pair of characters was analyzed. It was found that the type corresponding to the irradiated parent, when present, constitutes a small minority of the yield from a mixed-infected bacterium. This finding contradicts the hypothesis of complete independence of replication and reassembly of the hypothetical units; units derived from the active parent appear to be favored. It also casts doubt on the interpretation of the "loss" of a locus in these experiments as resulting from its specific inactivation, since a locus might be missing from the yield without necessarily being directly damaged.

The lack of certain proof for a localization of the ultraviolet-produced damage in any specific genetic determinant

of the phage particle leaves the interpretation of the phenomenon of multiplicity reactivation open. The occurrence of photoreactivation, discussed by Dulbecco, without contradicting the hypothesis of localized damage in discrete determinants, suggested the need for caution in interpreting multiplicity reactivation, since physiological mechanisms of repair may be involved. In an attempt to ascertain the role of genetic recombination in multiplicity reactivation, a group of experiments was done by Dulbecco using mixed infection with one irradiated and one active particle differing by two genetic characters. The mixed yields from such bacteria always contained more recombinant types than would be expected if the irradiated particle had to be repaired as a whole before taking part in recombination. This indicates that recombination actually is a primary factor in reactivation; there is a greater recovery of parts of an irradiated particle than of the particle as a whole.

[Evidence obtained while this paper was in press (Dulbecco, '52) has made the hypothesis of reactivation by genetic recombination inadequate and has led to its abandonment.]

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EXPERIMENTS ON PHOTOREACTIVATION OF INACTIVE BACTERIOPHAGES

RENATO DULBECCO
Department of Bacteriology, Indiana University, Bloomington

The photoreactivation of phage inactivated by ultraviolet light, observed accidentally in the course of experiments with a different purpose, belongs quite probably to the same group of phenomena as the light reactivation of streptomyces spores and bacteria discovered by Kelner ('49). While photoreactivation of spores and bacteria occurs in material previously exposed to ultraviolet, illumination of inactivated bacteriophage particles is without effect; photoreactivation takes place only if phage particles and sensitive bacteria are both present during exposure to visible light.

The availability of a system in which the phage component is inactivated by the ultraviolet light and the bacterial component acts as a reactivating agent is of interest, because the photoreactivation system can be dissociated into two parts which can be analyzed separately.

In order for reactivation to occur, inactive phage must actually be adsorbed on sensitive bacteria during the exposure to light. This is proved by the following experiment.

Irradiated phage and bacteria are mixed, in a condition which allows only very little adsorption (low sodium chloride content), and the mixture is exposed to visible light. After a light exposure that would give very high photoreactivation of adsorbed phage, the unadsorbed phage particles are separated from the bacteria by centrifugation. Assay of the free phage by plating in the dark shows that the unadsorbed phage has not been reactivated.

Exposure of sensitive bacteria to light immediately before infection with inactive phage does not cause reactivation if the subsequent infection is carried out in the dark.

The action spectrum of the reactivating light shows a definite band ranging from 3100 to about 4800 Å. Shorter wave lengths cannot be tested because of their inactivating power; longer wave lengths have no detectable effect. The maximum of the band is between 3600 and 4000 Å. This band presumably represents the absorption spectrum of the pigment responsible for adsorption of the reactivating light; its knowledge may therefore be a useful lead for the identification of the pigment.

A group of experiments was carried out to investigate the kinetics of photoreactivation. For this purpose the amount of photoreactivation is defined as

$$\frac{\text{increase in active phages after exposure to light}}{\text{number of adsorbed inactive phages}}.$$

Starting the exposure to light at the very moment of the infection and determining the amount of photoreactivation after different intervals of time (that is, for different doses of reactivating light), we observe an increase of photoreactivation concomitant with increasing dose. For bacteria exposed in nutrient medium, no further increase is obtained after about 20 minutes at 37°C. but, if resting bacteria are used, the increase continues for almost 100 minutes. The increase of photoreactivation with dose is almost linear for low doses; with high doses photoreactivation reaches a maximum which is dependent on the dose of ultraviolet used for inactivating the phage. The same maximum is reached by giving the same dose of reactivating light with various intensities in different lengths of time.

This behavior suggests that not all the inactive phage particles can be reactivated, and that the increase in photoreactivation with light dose reaches its maximum when all the reactivable particles are reactivated.

For low intensities of reactivating light, the amount of photoreactivation obtained with a given amount of energy in

one continuous exposure is independent of the light intensity. The amount of photoreactivation obtained with a given time of exposure (photoreactivation rate) is proportional to the light intensity. For high light intensities, the photoreactivation rate increases more slowly than the intensity and tends to a maximum, indicating the existence of dark reactions in photoreactivation with corresponding saturation.

Several factors influence the amount of photoreactivation obtainable with a given dose of reactivating light. Phage T2 inactivated with different doses of ultraviolet shows different amounts of photoreactivation. If we measure the survival of ultraviolet-irradiated phage both in the dark and after exposure to the light that gives maximum photoreactivation, and we plot the log survival against ultraviolet dose, we obtain two diverging straight lines. The difference between the two slopes can be used as measure of the photoreactivability of a phage, and offers a measure for comparison of the photoreactivability of different phages. The coli phages of the T system rank in order of decreasing reactivability as follows: T1, T2, T6, T3-T7, T4, T5.

Phages inactivated with different agents show very different photoreactivation. After X-ray inactivation there is very little photoreactivation. Phage inactivated with different ultraviolet wave lengths also shows differences in photoreactivation.

Photoreactivation is strongly influenced by temperature; the Q_{10} is not constant, varying between 8.3 for temperature between 3° and 13°C. and 1.86 between 27° and 37°C. This dependence of Q_{10} on temperature indicates a complexity of the reactions and perhaps enzymatic reactions.

Photoreactivation can be obtained at temperatures at which phage growth does not occur (+ 1°C.) and in resting bacteria in absence of any substrate. Photoreactivation is, therefore, conditioned by some early phase of the relationship between host and virus, and is independent of phage growth.

A group of experiments, still in progress, were undertaken in order to understand the relations between photoreactivation

and the multiplicity reactivation, which can take place in the dark. Important differences exist between the two types of reactivation. Photoreactivation occurs in bacteria infected with any number of phage particles, while multiplicity reactivation occurs only in bacteria infected with at least two phage particles. Some phages that show very strong photoreactivation show little multiplicity reactivation, for example T1, and phages capable of strong multiplicity reactivation, for example T4, are photoreactivated to a very small extent; T2 shows a high reactivability both by light and by multiplicity.

On the other hand, a connection exists between the two types of reactivation. Some experiments show that, under the influence of light, the collaboration among inactive particles inside the same bacterium, which leads to production of active phage, is considerably enhanced.

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CONTENTS

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